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PYROLYSIS GAS-LIQUID CHROMATOGRAPHY APPLIED TO A STUDY OF VARIATION IN ARTHRODERMA TUBERCULATUM

by

JAMES DWANE BROSSEAU

A THESIS

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EDMONTON, ALBERTA

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recomend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled PYROLYSIS GAS-LIQUID CHROMATOGRAPHY APPLIED TO A STUDY OF VARIATION IN ARTHRODERMA TUBERCULATUM submitted by James Dwane Brosseau in partial fulfilment of the requirements for the degree of Master of Science.

J.W. Cammichael
Supervisor

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Date 29 October 1974

ABSTRACT

Replicates of whole colonies of four species of closely related dermatophytes were analysed by pyrolysis gas-liquid chromatography (PGLC). The four species included fifteen strains of *Arthroderma tuberculatum*, and two strains each of *A. benhamiae*, *Nannizzia gypsea* and *N. incurvata*.

The chromatograph used was a Carlo-Erba fractovap model GV, equipped with dual hydrogen-flame ionisation detectors, automatic temperature programing, electronic signal attenuator, a variable-speed strip chart recorder, capillary columns (0.25 mm i. d. x 50 m), and an inlet-port carrier-gas splitter. The column was coated with 8% Carbowax 20M. A Carlo-Erba pyrolysis unit was used.

Dried homogenized fungal samples in glass capillary tubes were placed in the pyrolyser coil and pyrolysed under a stream of helium carrier gas for 20 seconds at approximately 800 C. Volatile hydrocarbons were sometimes added to the samples before pyrolysis. The time for the entire run was about 60 minutes, including an initial isotherm at 60 C for 12 minutes and a temperature program from 60 to 200 C at 4 C/min., and a final isotherm at 200 C for 10-15 minutes. The efficiency and resolution of the column were checked periodically throughout the course of the study.

Individual peaks on different pyrograms were identified as homologous with the aid of internal markers by the superimposition of pyrograms. Homologous peaks were given identical identification numbers. Retention time for each peak was recorded along with its peak height. Both data were analysed for variation. Usually 3 to 4 replicates were analysed for each fungal strain. The qualitative and quantitative comparisons of pyrograms of these replicates helped to confirm the limitations of PGLC as a technique for application to the taxonomy of fungi. Some of the limitations found were as follows: Not all of the components of the pyrolysates were resolved into individual peaks. The use of capillary columns failed to substantially increase the resolution over packed columns. It was found necessary to use larger than usual sample sizes along with a carrier gas-splitter in order to introduce enough pyrolysate through the inlet port into the mouth of the column. Variation in baseline within and between pyrograms occurred as a result of excessive column bleed-off and the elution of accumulated tars and high molecular weight components on the column. Baseline drift was compensated for by measuring the peak heights as the distance between the apex of the peak and the mid-point of the base inflection points. Variation in sample size which primarily caused variation in the mean peak heights between pyrograms was accounted for by dividing the height of each peak by the mean of all the peak heights within the same pyrogram. Variation in retention time of

peaks occurred mostly because of variations in temperature program and gas flow rates. The variations from the mean retention times of the markers were used as a correction factor to correct for the variation in retention time of neighboring peaks. It was thus possible to identify peaks on different pyrograms as homologous, with minor exceptions by comparing only the corrected retention times. It was also possible by this procedure to detect the presence or absence of peaks.

The peak height data extracted from the pyrograms of the fungal samples were analysed to compute average similarities between pairs of pyrograms. The average was calculated with each peak weighted equally. It was then repeated with each peak weighted for its information content assuming equal intra-strain variation, and with each peak weighted for information content using the average intra-strain range. The results of cluster analyses of the three sets of proximities were generally similar. Most, but not all, replicates of each strain were similar enough to be clustered together. Some strains belonging to the same species were also similar enough to be grouped in one cluster. Other strains of a single species varied sufficiently to be put in separate clusters. The nearest neighbor to each OTU (pyrogram) was always a replicate of the same strain.

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INTRODUCTION

Classically taxonomy of the fungi has been based on the morphology of sexual and asexual reproductive structures, and more recently their methods of spore production.

The Fungi Imperfecti (or Deuteromycotina) include the dermatophytes, fungi of medical and veterinary importance. The discovery of the perfect or sexual stages of some species of dermatophytes by Nannizzi (1933) and their recent rediscovery by Griffin (1960) and Stockdale (1961) and others has resulted in their reclassification as Ascomycetes, family Gymnoascaeceae, genus Nannizzia or genus Arthroderma.

Highly sensitive analytical techniques have been applied to fungal taxonomy and in particular the fungi imperfecti. Shechter et al. (1966) analysed the elctrophoretic non-dialyzable protein patterns from culture filtrates of Microsporum gypseum, Microsporum canis, Trichophyton mentagrophytes, Trichophyton tonsurans, Trichophyton rubrum, and Epidermophyton floccosum. Shechter et al. (1968a,b) extended their previous study to include four different isolates of the same dermatophyte species and some 15 new ones. Comparisons were made from data obtained from gel densitometer tracings. In general the variation was greatest at the interspecific level when compared with

the intraspecific variation.

In the majority of cases, the two mating types of dermatophytes cannot be differentiated by morphology alone. However, Rippon (1967) found that the enzyme elastase was produced by only the (+) mating type of the ringworm fungus Microsporum fulvum.

Rippon and Garber (1969) attempted to assess pathogenicity as a function of mating type and found that the mating types of some species of Gymnoascaeceae behaved differently in their pathogenicity. Since then Rippon (1971) found them also to have different proteolytic enzymes.

Studies were recently undertaken by Sekhon et al. (1974) to determine whether (+) and (-) mating type strains of Arthroderma tuberculatum could be differentiated from each other on the basis of electrophoretic patterns of general proteins, peroxidase, esterase and polyphenoloxidase. The number of protein and peroxidase bands was greater in the (+) strains 3181, 1905, 1967 than in the (-) strains 3182, 1593 and 1724. The esterase number was greater in the (-) mating type strains, while no polyphenoloxidase activity was found in either (+) or (-) strains.

Sekhon and Carmichael (1972), Carmichael et al. (1973),

and Sekhon and Carmichael (1973) examined species belonging to Nannizzia, Arthroderma and Microsporum by pyrolysis gas-liquid chromatography (PGLC), a technique introduced by Reiner (1965) to detect, characterize and classify bacteria by visual examination of their pyrolysis patterns (pyrograms). The pyrograms of the strains included in each of these studies demonstrated a marked difference between the mating types in some species, but little difference in other species.

In general these preliminary findings indicate that PGLC and other recent highly sensitive analytical techniques can provide new insights into variation among strains of the same species and between related species.

LITERATURE REVIEW ON GAS CHROMATOGRAPHY

This review concentrates on those aspects of gas-liquid chromatography and pyrolysis-GLC that are pertinent to its use in the production of data for taxonomic purposes. GLC and PGLC have many other applications and a vast literature spread through publications in different disciplines.

James and Martin (1952) were the first to introduce the technique of gas-liquid chromatography by applying it to the separation and subsequent identification of fatty acids. Many reviews treating various areas and applications of GLC are available, for example; Hardy and Pollard (1959), Hobbs (1960), Schomburg (1964), Dal Nogare and Juvet (1968), Leathard and Shurlock (1970), and Juvet and Cram (1974).

Initially GLC analysis was used primarily to identify unknown volatile compounds by comparing their retention time with that of known compounds. More recently GLC has been used to characterize complex samples of biological origin.

Abel et al. (1963) demonstrated the feasibility of discriminating between different organisms on the basis of their GLC pattern. Chromatograms of the fatty acid methyl esters of the lipids extracted from selected representatives from the families Enterobacteriaceae, Bacillaceae, Micrococcaceae and Parvobacteriaceae were analysed and

compared. Components were identified by comparing the elution times of esters of known composition. Similarities and differences in relative amounts of the carboxylic acids were noted among the selected species of the Enterobacteriaceae.

Soon thereafter, Brown and Cosenya (1964) in a similar study distinguished between Gaffkya and Micrococcus of the family Micrococcaceae on the basis of their fatty acid spectra.

Yamakawa and Ueta (1964) analysed species of Neisseria for cellular fatty acids and found a variety of constituents common to all except Neisseria haemolysans.

Bassette and Claydon (1965) analysed head space vapors produced by pure cultures of bacteria growing in milk. Using GLC they were able to differentiate amongst several species of Lactobacilli, Streptococci, and Aerobacter aerogenes, Escherichia coli and Achromobacter lypolyticum. Markedly different chromatographic profiles were obtained with these microorganisms of widely different morphological and biochemical characteristics. Profiles of high similarity, but with some differences, were obtained with closely related microorganisms.

Huis et al. (1966) examined bacterial volatile products or volatile derivatives of products synthesized during

growth by 29 strains of 6 species of Bacillus, Escherichia coli, Aerobacter aerogenes and Pseudomonas aeruginosa. A signature or fingerprint for each bacterium was established by arranging peaks corresponding to decreasing peak areas. Identification of the volatile metabolites was facilitated through the use of a number of known bacterial metabolic products. Statistical analysis of differences in chromatograms demonstrated the distinguishability of genera, species and strains based on either the quantitative or qualitative makeup of the chromatographic patterns.

One of the main advantages of gas-liquid chromatography is its rapidity of analysis of complex mixtures of volatile substances. Its extreme sensitivity has been demonstrated by Mitruka and Alexander (1968) who detected substances in the nanogram and picogram range with flame ionisation and electron capture detectors. The GLC instrument possesses great versitility through the appropriate choice of stationary phase or column coat, column dimensions, detector systems and instrument operating conditions.

A collection of gram-negative, non-sporulating organisms, including corroding bacilli, was divided into four groups by Prefontaine and Jackson (1972) on the basis of a gas-liquid chromatographic analysis of the esters of their cellular fatty acids.

As a result of these first publications, gas-liquid

chromatography began to be recognized as a potential means of assisting in the characterization of microorganisms.

These techniques plus those of more recent origin show one or another of four major analytical trends.

One: the chromatographic detection of specific metabolites produced by different microorganisms, as in Drucker (1970); Mitruka et al. (1970); and Brooks et al. (1972).

Two: the chromatographic characterization of structural components using derrivatives of extracted cellular components, as in Steinhauer (1967); Ellender et al. (1970); Theon et al. (1971) and Blashy and Zimmerman (1971).

Three: the analysis of derivatives of whole cell hydrolysates by Farshy and Moss (1970); Meyer and Blazenic (1971); and Wade and Mandle (1974).

Four: the application of pyrolysis to whole organisms or selected non-volatile components. The combination of gas-liquid chromatography and pyrolysis was first suggested by Zemany (1952) as a general approach to the identification of organic materials. He postulated that the identity and relative amounts of the various pyrolysis products from a given material should always be the same, just as the identity and the relative peak heights of the ions together

are the fragmentation patterns of a compound in a mass spectrometer.

Pyrolysates of organic molecules were first analysed for identification purposes and applied to GLC by Davison et al. (1954). They emphasized that the pattern of the peaks in a chromatogram of pyrolysates provided a characteristic "fingerprint" which could be used as a means of identification of the original substance.

Janak (1960) pointed out the considerable analytical potentialities of GLC analysis of pyrolysis products of non-volatile or poorly volatile substance such as naturally occurring macromolecules or polymeric products. He studied the pyrolysates of simple, structurally related barbituric acid derivatives and concluded that the primary pyrolytic fragments of the molecules present are quantitatively reproducible and qulaitatively highly specific.

Winter and Albro (1964) described a technique for the study of amino acids by gas chromatography of their low temperature pyrolytic products. They suggested that this technique may be applied to the identification of specific proteins and other nitrogenous materials.

Vollmin et al. (1966) displayed graphically the results of amino acid pyrolysis as peak heights and peak retention distance. Pyrolysis of amino acid mixtures produced

chromatograms which corresponded to the addition of pyrochromatograms of individual amino acids. Similar results were obtained in the fragmentation of peptides.

As in standard GLC studies, sample identification by pyrolysis-GLC evolved from comparison of the pyrograms of the sample under test with those of known materials

Chromatography of bacterial pyrolysates or substances of biological origin was suggested by Oyama (1963) as a technique for life detection experiments in extraterrestrial environments.

Reiner (1965) made the first attempt to classify related bacteria by their pyrochromatograms. He analysed 18 different antigenic strains of Escherichia coli, 4 types of Group A Streptococcus pyrogenes, 10 different pathogenic and non-pathogenic forms of mycobacteria and one strain of Shigella boydii. He found that each strain produced its own unique pyrochromatogram. He also found that these pyrochromatograms enabled him to detect reproducible similarities and differences such as slight but significant inversions of peak height ratio. The findings indicated a new source of data for biological taxonomy.

A review of pyrolysis gas chromatographic literature up until 1966 was compiled by Levy (1966).

Reiner (1967) extended the use of PGLC to include a wider variety of bacteria as well as fungi, mycoplasmas, mammalian cells and plant seeds. In this study the variation in retention times and peak area ratios among replicate and duplicate runs was found to range from zero to a few seconds and from 0.1% to 3.4% deviation from the mean respectively. Variations in pyrolytic profiles were found to be primarily quantitative; reflecting differences in composition of organisms at the generic, specific and in cases sub-specific levels. Comparisons were made by superimposing pyrochromatograms and noting differences.

Oyama and Carle (1967) analysed the pyrochromatograms of a number of species belonging to different genera using relative peak height ratios of selected peaks as a basis for similarities. Peaks were identified by their retention times relative to certain characteristic peaks in each pyrochromatogram. Striking similarities were noted among the bacterial pyrochromatograms as well as between the pyrochromatograms of microorganisms and those of bovine serum albumin and insulin.

Reiner and Ewing (1968) confirmed Reiner's previous work indicating that subspecific differences among diverse microbial taxa could be detected by means of PGLC of their whole cells. Attempts made to directly associate pyrograms of smooth cultures of Escherichia coli and Shigella with serological findings were not always sucessful. In some

cases, however, they were able to detect consistent differences in pyrograms between two Escherichia coli cultures differing only in their flagellar H-antigens.

Myers and Watson (1969) detected differences in pyrograms between rusted, mildewed, viral infected and healthy leaves using a technique similar to Reiner's (1967). They found relatively little variation among leaves of the same species.

A comparative study of desert soils, Precambrian shales and meteorites was undertaken by Simmonds et al. (1969) to distinguish biological material from fossil or meteorite organic matter using PGLC coupled with mass spectrometry. A representative series of pyrolysis fragments from major classes of bio-organic material such as polypeptides and free amino acids, carbohydrates, lipid, nucleic acid and hydrocarbon were also analysed by mass spectrometry. The results with proteins were in agreement with similar work performed by Vollmin et al. (1966) and Merritt and Robertson (1967). Proteins, peptides and amino acids yielded characteristic nitriles upon pyrolysis while carbohydrates degraded to a series of aliphatic aldehydes, ketones and furan derrivatives. Fats yielded unbranched alkanes and alkenes. No series of characteristic products was found for nucleic acid pyrolysis however. It was also found that several individual pyrolytic peaks were made up of more than one component.

Reiner and Kubica (1969) demonstrated the classification of species and strains of mycobacteria using PGLC. Profiles of certain characteristic peaks at the high temperature end of the analysis enabled them to demonstrate differences as well as similarities among the species and strains.

Reiner et al. (1969) also suggested PGLC as a diagnostic tool for the identification of mycobacteria. Small differences were noted between pyrograms of drug-resistant and drug sensitive strains of Mycobacterium tuberculosis.

Cone and Lechowich (1970) found that differences detected between between pyrochromatograms of cultures of Clostridium botulinum types A, B and E were suitable for type identification but did not differentiate between strains of the same type. Pyrograms permitted differentiation between spores and vegetative cells.

The pyrolysis gas-liquid chromatographic-mass spectrometric analysis of Micrococcus luteus and Bacillus subtilis was reported by Simmonds (1970). The mass spectra of individual pyrolysis products were identified by comparison with reference spectra. Simmonds found a close qualitative similarity between pyrolysates of the microorganisms and those of other biological materials,

partly reflecting the common presence of proteins, and carbohydrates. Some of the major pyrolytic products were phenylacetonitrile, benzene, toluene and acetamide. Acetamide was identified as the most abundant thermal fragment in the microbial pyrolysates. The major sources of acetamide were suggested to be glutamine, asparagine and acetyl muramic acid. Some pyrolysis fragments were found to be unique to one organism only.

The gas chromatographic behavior of the polyene antiobiotics candicidin, levorin and trichomycin after low and high temperature pyrolysis was investigated by Burrows and Calam (1970). Similarities and differences in pyrograms upon visual inspection were related to the similarity in the antiobiotics' basic structural makeup and to variations in biological potency.

Vincent and Kulik (1970) analysed three strains each of four species of the Aspergillus flavus Group for differences at the species and strain levels using PGLC. A common reference peak found in all pyrograms was used to correct for variation in retention times. Any two peaks were considered homologous if their retention times were within 1% of each other. Composites of the pyrograms were formed as bar graphs representing particular species. The number of homologous peaks present in pairs of representative species was used to calculate % similarity from a modified Sokal and Sneath (1963) formula. Four fungi of the

Aspergillus flavus group were differentiated to the species and strain level. Their findings supported those of Kulik and Brooks (1970) who performed a similar study using disc gel-electrophoresis of whole cell soluble proteins.

A range of biochemical and biological samples were examined by Myers and Smith (1972) for similarities using PGLC. Pyrolytic patterns of biological samples were in many cases related to their known biochemical composition. Peaks in the pyrograms were identified by their retention times while relative peak heights reflected the quantitative aspects of the pyrograms. The greatest degree of similarity among the biochemicals was between proteins and nucleic acids.

The feasibility of using computers to compare bacterial pyrograms was tested by Menger *et al*. (1972). Library computer files contained contained the peak retention times and amplitudes of six to nine peaks from six bacteria, each belonging to a different serological group of *Salmonella* species. As in previous work by Reiner and others, the complicated initial portion of all pyrograms was ignored. Ten unknown bacteria were examined and nine were correctly identified by computer. Deterioration of the chromatographic column however, led to changes in the absolute values of the pyrogram data.

Meuzelaar and Veld (1972) found that the level of

reproducibility was sufficient to superimpose pyrograms of the same strain of Neisseria. Pyrograms of different strains however, demonstrated differences in relative peak heights.

Fifty-four coded samples representing 47 Salmonella species were correctly sorted and classified by comparison of their PGLC tracings by Reiner et al. (1972).

Similar studies on mammalian cells and a variety of normal primary cells were performed by Reiner and Hicks (1972a). Reiner and Hicks (1972b) provided preliminary evidence on the applicability of PGLC as a diagnostic tool in detecting inherited chromosomal and biochemical disorders in fibroblasts cultured from skin tissue.

Sekhon and Carmichael (1972) examined dermatophytes belonging to Nannizzia, Arthroderma and Microsporum. They found that low resolution chromatography was not suitable for separating those fungi. Further work by Carmichael et al. (1973) using chromatographic columns of higher efficiency, produced a threefold increase in the number of resolved peaks. Pyrogram data was entered onto disk files through a computer terminal and analysed for relative proximities and clusters using the TAXMAP classification program of Carmichael and Sneath (1969) and Carmichael (1970). When peak height response was recorded on a 0-1-2 peak height scale, the cluster analysis provided a stable

classification with clusters well separated compared to the variation within them.

In further work using the same techniques, Sekhon and Carmichael (1973) analysed five strains of Nannizzia fulva and nine of Nannizzia cajetani for variation between strains based on their pyrolytic patterns. The variation between pyrolytic profiles of strains was small compared with those between the two species. The cluster analysis separated some strains on the basis of their mating type, while others were found in the same cluster.

Reiner et al. (1973) indicated the potential use of PGLC for establishing a more stable classification system for Leptospira. Twelve authenticated strains were differentiated and identified by their pyrolytic profiles.

Similarity values were calculated by Vincent and Kulik (1973) for strains of the Aspergillus glaucus group by intepretating and comparing the number of pyrolytic chromatographic peaks in which two strains agreed or disagreed. These data indicated a close correlation with conventional taxonomic placement of the taxa.

Three strains each of nine Penicillium species were analysed for variability or similarity based on data from pyrograms by Kulik and Vincent (1973). Similarities were claculated in the same manner as Vincent and Kulik (1973).

On the average, intraspecific similarities were higher than the interspecific similarities, but there were individual exceptions.

The use of PGLC as a chemotaxonomic method for insects was evaluated by Hall and Bennett (1973). Species of cockroaches were easily identified based on species specific peaks or peak groups found in the high temperature portion of the pyrograms. Samples were spiked before pyrolysis with n-alkanes and the retention times were calibrated relative to the n-alkane internal markers.

A streptococcal strain was differentiated from its mutant lacking the type III polysaccharide antigen by Veld et al. (1973). Differences were observed in pyrograms of whole cells, purified cell envelope fractions and purified cell wall antigens. The differences in relative peak heights also aided in discriminating between other known streptococcal polysaccharide type antigens.

Haddadin et al. (1973) were able to differentiate Vibrio cholerae from the nonvibrios Aeromonas, Pseudomonas, Escherichia, Salmonella, Shigella, and Proteus on the basis of a single individual peak found in the pyrograms of the vibrios. Differentiation was also made among 45 Vibrio cholerae strains allowing classification into 3 distinct categories, not in total agreement with conventional groupings.

The potential of PGLC as a chemotaxonomic tool was evaluated by Seviour *et al*, (1974) in studies on *Eucalyptus* mycorrhizas. The analysis of peak heights as a simple quantitative measure of dissimilarity was described. Experimental variability between replicate samples was found to limit the taxonomic significance of the study.

Qualitative analysis in gas chromatography consists basically of comparing the retention time of an unknown component with that of a known. Qualitative use might also involve the combination of GLC with instrumental techniques such as mass spectrometry, infrared spectroscopy and others. Quantitative analysis revolves around data acquisition from chromatographic peaks in the form of peak heights or peak areas and its use to determine certain quantitative aspects of the sample material.

The basic aspects of qualitative chromatographic analysis have been reviewed by Harrison (1957); Hardy and Pollard (1959); McWilliam (1961); Schomburg (1964); Perry (1967) and Arakelyan and Sakodynskii (1971).

The accuracy and reproducibility of gas chromatographic analysis is dependent upon factors such as type of detector, operating conditions and method of analysis. The three types of detectors most commonly in use are: thermal conductivity, flame ionisation, and electron capture

detectors (see Mitruka and Alexander, 1968). Flame ionisation detection is discussed in papers by Bocek and Janak (1971), Keller (1973) and Blades (1973).

Many aspects of the GLC technique, such as precision, time required for analysis, errors in recorder performance, area and peak height determinations, and human factors are discussed by Ettre (1965); Harris and Habgood (1966); Kuksis (1966); Carter (1967); Leathard and Shurlock (1970) and Lyons (1970). Derge (1972) published a two part article in which the effect on the reliabilities of sample preparation, sample introduction, detector design and electronics are reviewed.

Detailed investigations on the precision and reproducibility of pyrolysis gas-liquid chromatography systems are found in Levy (1966,1967); Perry (1968); McKinney (1969); Stack (1969) and Jones (1969).

The reproducibility of pyrograms produced in two different laboratories using different pyrolysis units was demonstrated by Andrew et al. (1963). Meuzelaar and Veld (1972) found their pyrolysis method suitable for the analysis of biological samples such as bacteria, but noted that further development would be necessary in order to achieve a useful degree of interlaboratory reproducibility. The further development of PGLC is described by Levy and Fanter (1969) and Merritt and Robertson (1972) as being

dependent upon the standardization of pyrolysis and experimental conditions. Problems concerned with reproducibility have been undertaken by a study group consisting of Perry (1969), Coupe et al. (1970), and Douglas (1971).

Several workers have looked into the reproducibility of results on different columns constructed in an identical manner. Reiner and Ewing (1968) found that pyrograms of a single bacterial organism pyrolysed and analysed on different columns demonstrated altered pyrolytic patterns. Similar results have been suggested by Meuzelaar and Veld (1972), Haddadin et al. (1973). Sekhon and Carmichael (1973) found that variation affecting a PGLC study of Strain variation in two species of Nannizzia was due to the use of newly constructed columns.

Preselected identical culture media were found to be necessary in order to obtain consistent and reliable pyrolytic patterns among replicates of bacteria. Oyama and Carle (1967) reported large differences in pyrograms of a fungal species Candida pulcherrima in malt extract and in trypticase soy broth, while similar media gave similar results.

Reiner and Ewing (1968) reported that changing the culture media from blood agar base to phytone yeast extract did not produce any changes in the pyrolytic profiles of

various microorganisms. Similar results were also found by Veld et al. (1973) analysing the pyrolytic patterns of streptococcal strains on four different media.

Cone and Lechowich (1970) also indicated that growth media had some effect on the pyrograms of clostridial species. Sekhon and Carmichael (1972) indicated that the type of medium influenced the pyrograms of cultures of Nannizzia gypsea grown on phytone yeast extract agar as compared to cereal agar.

METHODOLOGY

Principles underlying Pyrolysis Gas-liquid Chromatography

Chromatography is a term used to describe all separation processes where the separation of the components is effected by their partition between a stationary (fixed) phase and a moving (percolating) phase which flows over the first.

Gas-liquid chromatography is a class of partition chromatography where the stationary phase is a liquid coated on the inert solid support of a packed column, or with open tubular capillary columns, the internal wall of the narrow tube is coated with the liquid phase. The mobile phase is a gas such as helium or nitrogen.

GLC can separate mixtures that are volatile or have been converted to volatile derivatives. Volatile mixtures are flash-vaporized upon injection into the heated inlet port of the chromatographic column. Non-volatile materials are first pyrolysed to yield volatile degradation products, by heating them in an inert atmosphere in a unit connected to the inlet port of the chromatograph.

Pyrolytic breakdown products are carried into and thru the column by a constantly flowing stream of nitrogen or helium gas. Separation of the components in the column is

achieved by repeated equilibration between the mobile gas phase and the stationary phase, based on the absorption, solubility and polarity of the column coat or stationary phase. Hence the components are slectively retarded by the stationary phase and when in the gas phase are carried towards the column outlets. The freeing of the molecules from the adsorbent can be temperature dependent. A high degree of separation can be achieved by varying the nature of the stationary phase and the length and temperature of the column. Temperature programing of the gas-liquid chromatographic column during analysis, permits both a good separation of the more volatile pyrolytic components at low temperature and a speedier elution of the less volatile components at higher temperatures.

A detection device records the emergence of molecular components from the column and the data is displayed on chart paper as a series of peaks. The area under each peak is proportional to the concentration of the particular component in the mixture, although peak height is also influenced by baseline drift. Many types of detectors are available. The flame ionisation dector consists of a combustible mixture of helium or nitrogen, oxygen and hydrogen which is burnt as a small flame. When a peak of organic vapor is passed into the flame the ions produced allow an increased current to flow between a pair of electrodes polarized with about 300 V.

A gas chromatograph may be operated in a dual column mode. That is, a second column is placed in the same temperature programmed oven to serve as a reference column. The use of maximum or near maximum temperature operating conditions predisposes the column to an increase in column coat bleed off or decomposition during each run. The detection of this material results in an increase or shift in the base line of the chromatogram. This effect is exagerated at relatively high detector sensitivities. The purpose of the second column and detector therefore, is to reduce the level of baseline drift detected from the primary column. This is accomplished when corresponding signals from the two columns are relayed into a compensating unit, which generates a signal based on the difference between the two detectors. Material which builds-up on the column from the previous pyrolysis chromatographic runs also causes baseline drift, but dual column operation does not compensate for it unless the sample and reference column are alternated with each successive run.

Identification of components can be achieved by comparing their retention time with that of known standards, or the contents of a particular peak can be isolated by means of a refrigerated trap and identified with infra-red or mass spectrometry.

<u>Peak identification and retention time variation</u>

Pyrograms consist of an array of peaks representing eluted pyrolytic components detected with a flame ionisation detector (FID) at the outlet of the chromatographic column. Theoretically, a given compound should elute from a given column at a specific time after injection, if the column operating conditions are held constant. This ideal is not achieved in practice. When pyrograms of replicate samples are superimposed and viewed by transmitted light, the peaks do not match exactly throughout the length of the run. Figure 6 shows three pyrograms with their pyrolysis times lined up vertically on the left side. The dashed lines connecting certain distinctive peaks illustrate the way that retention time may drift to the left or the right in different parts of the run. This drifting is likely due to minor variations in operating conditions such as gas flow rate or column temperature. Even though there is some variation in absolute retention time, it is assumed that components will still be eluted in the same order, regardless of minor variations in operating conditions. Thus it has become customary to use relative retention time to identify different peaks. Relative retention for a pair of substances is the ratio of their net retention volumes(product of retention time and gas flow), usually expressed as a number larger than unity. In isothermal chromatography, relative retentions are often expressed as a fraction of the time required for elution of the last major component in a sample. In programmed-temperature gas chromatography, drifting in retention time has been observed

to occur at different rates in different parts of the curve. In previous studies, characteristic peak patterns have been used as calibration markers to adjust the retention times of portions of the pyrogram, thus correcting for drift. However, it is known that single peaks may be mixtures of components, and it is not possible to tell whether differences in retention time of a characteristic peak are due to drift or to a real difference in retention due to differences in composition. This becomes especially problematical when comparing pyrograms of strains that differ widely and where some peaks may be absent from one or the other pyrogram. An attempt to alleviate this problem was made by the use of added volatile markers of known composition and hence supposedly constant elution time. The variation of these markers in different runs should indicate the degree of drift of neighboring peaks and aid in the identification of of homologous peaks in different pyrograms. Internal markers might also aid in the comparison of inter-column or inter-laboratory pyrolysis data.

Column efficiency and resolution

Measurements of column efficiency and resolution are obtained under various operating conditions. Such measurements are used to evaluate the column coating technique in comparison with published data and to check on column aging effects and general gas chromatographic

conditions over the column lifespan.

The efficiency of a column is expressed as the number of theoretical plates or equilibration stages, which is a measure of the relative broadening of a chromatographic peak expressed at constant temperature as a function of carrier gas velocity. The number of theoretical plate equivalents (n) can be calculated from the chromatogram, according to:

$$n = 16(t^2/Wb^2) \text{ or } n = 5.54(t^2/Wh^2),$$

where t = retention time, Wh = peak width at half height, Wb = peak width at base intercept, see Fig. 1. The length or height equivalent to one theoretical plate (HETP) can be calculated from the column length (L) and (n) : HETP = L/n. When the plate height is large, the chromatographic peaks are wide relative to their heights.

The efficiency for the separation of a particular pair of components is defined according to Ettre (1965) by resolution (r) :

$$r = 2(t_2 - t_1) / Wb_1 + Wb_2$$

where (r) is determined by the relative position of two consecutive peaks in a chromatogram, see Fig. 2. Open-tubular capillary columns were chosen to separate pyrolytic components of microorganisms with the hope that it would increase the number of resolved peaks as a result of the columns higher efficiency potential over packed columns. Higher resolution would also permit more reliable peak

identification, since fewer peaks would be composed of mixtures of components.

Computer aided analysis

1: Calculation of relative proximities.

The relationships among strains of Arthroderma tuberculatum and other closely related species were analysed with respect to the relative similarities of their pyrograms. Data in the form of peak retention times and peak heights were extracted from the pyrograms in numeric form. The peak heights were then used to compute relative proximities or differences between pairs of pyrograms or OTU's (operational taxonomic units). The TAXMAP classification program as developed by Carmichael (1970) sets out to evaluate statistical relations among items or OTU's (pyrograms) represented by a set of variables or attributes (peaks). The attributes are useful for classification if they are comparable for any pair of OTU's on a one to one basis, that is, if they are homologous attributes.

Several other assumptions may be made about the relative information content of each attribute. The first is that each attribute can be described by a scale upon which the state of each OTU can be coded as a single unique position or value (peak height). Secondly, each observed

attribute value is an approximation of the true position of the OTU on that scale. The uncertainity associated with the OTU's position on the attribute scale arises from variation inherent in the measuring procedure or in the population measured. Lack of complete separation from the influence of other attributes may cause additional uncertainity. Thirdly, over a given scale, at a given confidence level, the confidence interval in scale units is constant, that is it is independent of the attribute value. Finally, it may be assumed that at a given confidence level, the relative information content for classification of any scale is the base 2 log of the number of contained classes, each one confidence interval wide, plus one if the confidence intervals for end classes are centered on the maximum and minimum scale values. For a continuous scale, where the attribute values are means of small numbers of measurements, one can assume that the average, over all the OTU's, of the ranges about the mean will be close to 4 standard deviations, and thus the average range can be taken as a 95% confidence interval. Where a large number of measurements have been made to determine each attribute value, standard deviations can be calculated in the usual manner, and their mean used to calculate the 95% confidence interval.

The relative distance (dij) between the states (V) of the ith and jth OTU's on a single attribute is taken to be quantified by

$$dij = \frac{|Vi-Vj|}{(Vmax - Vmin)},$$

that is, it is the difference between the observed values divided by the observed range over all the OTU's. The relative distance (Dij) between the iTH and jTH OTU's based on a number (n) of attributes is taken to be the weighted (W) arithmetic average of their relative distances on each attribute,

$$Dij = \frac{\Sigma[(dij) \times Wk]}{\Sigma Wk} \qquad k=1 \text{ to } n.$$

The TAXMAP program provides either for weighting all attributes equally, or for weighting them according to their relative information content.

2: Cluster analysis.

When relative proximities or differences between pairs of OTU's have been calculated for each attribute and the average taken over all attributes, the program judges whether some OTU's are sufficiently related to each other and sufficiently unrelated to others to constitute clusters. The procedure for judging clusters is as follows. The proximities between the pairs of OTU's are rank-ordered, the

closest first. A five percent 'tail' is deleted from the top and the bottom of the rank ordered list. The range of the remaining distances is determined and divided by 10 to yield a distance called CUT. This distance is added to the smallest distance in the middle 90% of the rank-ordered list to yield a distance called QUIT. Pairs of points closer than QUIT, if they if they are the closest pair neither of which is already allotted to a cluster, are considered to form the nucleus of a cluster. The closest point to any already in the cluster is then added to the cluster unless a) its distance from the closest OTU is greater than QUIT and its distance minus the average of the best and the worst of such previous distances is greater than CUT, or b) its average distance from all the previously admitted points is greater than QUIT and the average for this point minus the average for the last previously admitted point is greater than CUT, or c) a ratio criterion, which prevents scattered points from bridging large elongated clusters, is not met, or d) the closest point is already a member of a previously formed cluster. OTU's which are farther than QUIT from any other OTU's and which were excluded from diffuse clusters by the above critera are considered to be single member clusters. A subroutine of the TAXMAP program produces a complete proximity matrix along with a histogram of the weighted average relative proximities at 0.01 intervals. This is followed by a nearest neighbor table listing the eight closest neighbors each OTU. The inter- and intra-cluster proximities are also printed out in the form of half

matrices, to assist in preparing a taxometric map.

3: Graphic display of cluster relations.

Most statistical methods for reducing dimensionality (such as multi dimensional scaling and factor analytic methods) produce some distortion of all the distance relations between the items. The TAXMAP mapping procedure, by successive triangulation, maintains the nearest distance relations without distortion, distorts some relations as specified in the map, and omits some of the relations. This method preserves what are assumed to be the most important relations in the classification.

Multidimensional scaling (MDSCAL)

MDSCAL was written by Kruskal (1964a,b,1969), and adapted for IBM S/360 by Carmone (1969), and distributed by the Marketing Science Institute, Cambridge, Mass. USA. MDSCAL is a computer program that constructs a configuration of points in space from information about the distances between the points. MDSCAL also uses a distance which is appropriate for Euclidean space. MDSCAL places points or items to be classified (OTU's) in a space of reduced dimensionality so as to minimize a stress function which measures the badness-of-fit between the configuration of points and the data. It finds the minimizing configuration

by starting with some configuration, and moving all points a bit to decrease the stress, then iterating this procedure over and over again until the stopping criterion is reached. We used MDSCAL to produce a "best fit" two dimensional configuration of the clusters detected by TAXMAP. The data entered into the MDSCAL procedure was the halfmatrix of center-to-center distances between the clusters, as shown in Table XI.

MATERIALS AND METHODS

Organisms

The cultures used included (+) and (-) mating types of; Arthroderma tuberculatum, Arthroderma benhamiae, Nannizzia gypsea and Nannizzia incurvata. The details of the strains are presented in table I.

Cultural conditions

The culture medium used was cereal agar, made from Pablum precooked mixed cereal, 100 g, agar 15 g, deionized distilled water, one liter.

Fungi were grown on cellophane membranes (52 x 52mm), which were placed on top of the medium in Petri plates (90mm) before inoculation. Petri plates were inoculated by transfering a bit of fungal growth from the periphery of 2-week old cultures maintained on cereal agar slants. After incubation for two weeks at 250 C, the colonies were removed from the culture medium, dried in a press, and stored in plastic envelops.

The procedures for growing, harvesting, and storing the fungal colonies are the same as described by Carmichael (1963), and Sekhon and Carmichael (1972).

Three of four replicate colonies were prepared for each strain. Samples prepared from the colonies of any one strain are referred to as replicate samples.

Sample preparation

The fungal growth on the cellophane was scraped off gently using the flat edge of a sterilized spearpoint dissecting needle and shovelled into a small tissue grinder, size AA (Kontes Glass Company N.J.). About one to two ml of solvent (ethanol:acetone:water, 50:20:30, v/v) was pipetted into the tissue grinder and the fungus homogenized in it to make a thick suspension or paste depending on the texture of the colony. The suspension was transfered into capillary tube pieces (1 mm internal diameter x 3.5-7 mm long, Kimble Products #34500, high melting point tube) either by holding the tube with forceps at one end, while the other end was dipped into the suspension, or by micropipetting the suspension into the sample capillaries with aid of a 100 microliter micropipette. The suspension was sufficient to yield two samples per colony. The dried sample stuck to the tube walls. Samples were found to range from 7 to 8 mg per tube. Initially each capillary tube used was preweighed before the sample was introduced into it, and then reweighed when filled with sample and dried. The range in size of each capillary tube was standardized at between 4.5 to 5 mm in length. The differences in weight among the tubes was found to be so small that preweighing of each tube was

dispensed with and the tare weight taken as the average weight previously found for empty tubes. Samples were stored for a minimum of a week in a covered sterilized petri plate for drying at room temperature.

When fungal samples were stored at room temperature for periods longer than two weeks, one or two peaks disappeared from their pyrograms. One peak would disappear sooner than the other over the first four week period after sample preparation. The first peak to disappear corresponded to the retention time of acetone under similar chromatographic conditions. The second peak to disappear corresponded to that of ethanol. Pyrolysis of the solvent used to homogenize fungal samples yielded two peaks with retention times corresponding to that of acetone and ethanol as in Fig. 5. Allowing the fungal samples to remain in the heated pyrolysis chamber for 10-15 mins. effectively removes those peaks from the fungal pyrogram.

GLC apparatus

The chromatograph used was a Carlo-Erba, model GV, equipped with a hydrogen flame ionisation detector unit. All samples were run on capillary columns (0.25 mm internal diameter x 50 m) coated with 8% Carbowax 20M (polyethylene glycol). Samples such as chromatographic pure benzene (Figure 1) (Fisher Scientific Company, N.J.) and Carbon-5 to Carbon-9 homologous hydrocarbons (Figure 2) (Fisher

Scientific) were analysed at a column temperature of 80 C. Ethanol and acetone were also chromatographed, but at 60 C. Further operating parameters of the instrument were: injection temperature, 260 C; detector temperature, 280 C; hydrogen and air pressure supplied to the flame ionisation detector units were 0.6 and 1.5 kg/cm² respectively; carrier gas, helium at an outlet flow rate of 1.8 ml/min; electronic signal input and output linear attenuation was 10^2 and 4 respectively; chart speed one-quarter and one-half inch per minute. About 8 ml of carrier gas was discharged through a carrier gas splitter during the one minute sample injection period, in order to have a sufficient gas flow to flush the pyrolysis products from the pyrolyser chamber to the entrance of the column.

Capillary open-tubular column coating

The capillary column consists of an empty tube in which the stationary phase is coated on the inside of the column walls. The column coating technique is essentially that described by Dykstra and de Goey (1958), as the dynamic coating method. It consists of filling the column tubing with a solution of the stationary phase and then emptying it under constant nitrogen or helium pressure. When Carbowax 20M was used as the stationary phase, column plugging would occur due to the presence of fine particles in the coating solution. Unplugging of the column was performed by the application of a gas pressure between 1000 to 2000 psi to

the column. Coated columns that had been unplugged in this fashion were not very efficient columns for chromatography. Centrifuging the coating solution only diminished the incidence of column plugging. Plugging of the column was completely avoided by filtering the coating material through sintered glass immediately before coating the column. Emptying the column of coating solution under constant pressure was necessary in order to obtain a uniform coat on the column internal surface. It was also necessary to expell the coating solution at a speed of no more than 1 drop/20 sec., in order to avoid variation in column coat thickness. A gas pressure of approximately 110 to 120 psi was found necessary to achieve this flow rate. Most commonly used pressure guages reading a maximum of 120 to 140 psi output do not in fact put out that amount of gas pressure. It was necessary to purchase a pressure guage with a maximum indicated output pressure of 200 psi in order to obtain a working range of within 100 to 180 psi.

The column coating technique used was as follows. The column was attached to the reservoir of about 3 ml of a solution containing 0.8 g of the stationary phase per 10 ml of methyl chloride.

The reservoir was attached to a nitrogen pressure of 160 to 200 psi, at a linear flow rate of 20 ml per minute. The column was filled with the stationary phase liquid. The column was then removed from the reservoir and attached

directly to the nitrogen gas source. A clean second column of equal dimension was attached to the outlet of the coat-filled column, a procedure similar to that described by Nikelly (1972). A helium or nitrogen pressure of 120 psi was restablished and maintained to coat the column at 55 C until the first amount of the coating liquid emerges from the apparatus. The column was then removed from its gas source and backup column and placed in the chromatographic oven for drying and conditioning.

Capillary column drying and conditioning

The column is attached to the inlet port of the chromatograph apparatus but is not connected to the detector. For several hours at 60 C a gentle stream of nitrogen at 15 psi was run through the column to evaporate the coat solvent.

Still mounted in the chromatograph the nitrogen flow is readjusted to about 2 ml per minute, or 22 psi. The column temperature is then programmed at 1-2 C per minute from 55 C to near the operating limit of the liquid phase repeatedly over one to two days. At the end of the conditioning period, the column is connected to the detector.

Capillary column cleaning

The column cleaning technique was essentially that

described by Prevot (1969) for stainless steel capillary columns. Plugging of the 0.25 mm i. d. column occurred when cleaning solutions were not filtered immediately before use with a sintered glass filter. Plugging was also found to occur when a too powerful solvent in a too large a volume was used as the first solution to pass through the column. The incidence of column plugging during cleaning was lowered by the application of a 1500 to 2000 psi tank of carrier gas connected directly to the column during cleaning. This also helps to speed up the cleaning process. Unplugging of a column can be accomplished by reversing the attachment of the column to the gas pressure tank. Unplugging usually occurred after the first attempt at this procedure.

All cleaning solvents and solutions were filtered twice through sintered glass #3 immediately before use. The source tank of nitrogen gas was between 1500 to 2000 psi. Columns are rinsed once with one ml of the solvent used in the original filling, in this case, methyl chloride. Then the columns are rinsed twice with the following 6 milliliter volume set of solutions (Fisher Scientific Company, N.J.): chloroform, xylene, acetone, distilled water, potassium hydroxide 10%, distilled water, methanol, chloroform and methylchloride. The columns are dried with a continuous flow of nitrogen gas.

Tests of column operation

The basis for the tests is described in Methodology and illustrated in Figs. 1 and 2. About one-half microliter of volatile samples such as ethanol, acetone, benzene or carbon-5 to 9 homologous saturated hydrocarbons was injected into the inlet port of the chromatographic apparatus through a preconditioned septum using a 10 microliter Hamilton syringe. The peak retention distances in millimiters and corresponding peak widths were determined from gas chromatographic runs at 80 C for the different gas flow rates generated by five different pressures. The efficiency was calculated from the chromatograms of benzene. Resolution was calculated from chromatograms of C-5 to C-9 hydrocarbons.

Pyrolysis apparatus

A Carlo Erba pyrolysis unit and chamber was used. This unit contains a coiled nickel-chrome filament which fits into the pyrolysis chamber. The chamber has its own inlet for supply of carrier gas which is preheated. Samples can be mounted or layered onto the filament itself. The pyrolysis chamber is flushed of air with carrier gas and then inserted, with the aid of an accessary needle attachment, into the septum-covered inlet port. The sample is pyrolysed for 20 seconds at a current of 2.3 A as determined by the control panel (Carlo Erba). Pyrolysis

temperature as judged by filament color is about 800 C. Capillary tubes containing fungal samples were placed inside the pyrolyser coil.

Initial operating conditions

Immediately before pyrolysis, 0.25 microliters each of methyl caproate (carbon six) and methyl undecanoate (carbon eleven) could be added to the sample tube as internal standards. Temperature programing was needed to shorten the analysis time. After an initial isotherm of six minutes at 65 C, the chromatographic oven was programed from 65 C to 180 C with a linear rise of 2 C per minute. When the upper limit had been reached, the run was continued isothermally until elution of pyrolysis products appeared to be complete. Operation was in dual column mode. The electronic signal attenuation was 10^2 input and 8 output for the initial isotherm, 4 output for the duration of the temperature rise, 2 output for the final isotherm. The program time was approximately 70 minutes, analysis time about 85 minutes. Chart speed was one-quarter inch per minute, starting at the time of sample pyrolysis.

Changes in operating conditions

The standard operating conditions previously described required an analysis time of approximately 75-85 minutes. It was desired to decrease the length of analysis time so

that more samples could be run per day and to provide earlier elution of those peaks at the high temperature end of the scale. This was achieved without reducing the number of peaks resolved by the following changes:-

1); The temperature programing rate was increased from 2 C/minute to 4 C/minute. 2); The carrier gas flow pressure was increased from 25 psi to 32 psi. 3); The initial isotherm was doubled in time from 6 to 12 minutes in duration and 4); the temperature of the initial isotherm was lowered to 60 C. The result was to decrease the run time to less than 50 minutes.

Various types of inlet port septa were utilized in an attempt to diminish excessive bleed and plugging of the injecting needle. None were found to be completly satisfactory. The solution was to eliminate the septum entirely. The pyrolyser chamber was instead connected directly onto the inlet port of the chromatographic column. The construction of the pyrolysis chamber already provided for such an attachment. The direct metal connection provided a further advantage; the pyrolysis chamber becomes heated by conduction transfer from the inlet port flow valve. This minimizes condensation of pyrolysis products on the chamber walls. Air trapped within the pyrolysis chamber was flushed out before pyrolysis by opening the sample-splitter valve wide open.

It was found that the 6-carbon marker, methyl-caproate, eluted at the midway point of the temperature program used, and the 11-carbon marker, methyl-undecanoate, eluted at the maximum temperature portion of the same program, as seen in Fig. 4. The undiluted markers were added as a drop from a 10 microliter Hamilton syringe, with the sample capillary already inserted in the pyrolyser coil. The pyrolyser was then immediately attached to the pyrolyser chamber and activated. A twelve minute isothermal period followed at which point the temperature program was started manually. The automatic isotherm-to-program procedure was not used because it included an automatic pre-isotherm-conditioner which allowed the markers to vaporize while waiting for sample pyrolysis. The program used shut itself off at the high temperature end of the run and then continued isothermally. The temperature increments on the Carlo Erba gas chromatograph temperature guage were large enough to make precise reading difficult. Errors in base temperature readings were considered a primary source of variation in peak retention times.

Capillary tubes containing samples of ethanol, acetone and the solvent were placed in the pyrolyser coil and volatilized with a column temperature of 60 C. The pyrogram of the solvent is demonstrated in Fig. 5. The reason for the pyrolysis of the solvent is that the fungal sample preparations contained amounts of the solvent detected in the fungal pyrograms, discussed under Sample preparation.

The stationary phase used to coat further columns was Carbowax 20M-TPA (Chromatographic Specialities, Ontario), a material similar to Carbowax 20M, but able to withstand continual operating temperatures of 200 C to 225 C instead of only 180 C to 200 C.

The range in sample size caused variations in the mean peak heights of replicate pyrograms. The use of a sample carrier gas-splitter helped to control the amount of pyrolysed material entering the column. It was first necessary to correlate various sizes of sample with the size of split. Slight deviations in the split size for a particular sample size would also cause the mean peak heights to vary.

Variations in sample size caused variations in peak heights. Normalizing for variation in peak heights between pyrograms was found necessary to minimize misleading statistical interpretations after analysis. Compensation for variation in sample size is described below. In addition, small sample size sometimes caused some of the smaller peaks to disappear between replicate pyrograms. Sample size variation was suggested by Levy (1967) as a cause for changes in relative heights of different peaks between replicate pyrograms. A decrease in the number of peaks along with alterations in relative peak heights would occur in pyrograms whenever the pyrolyser coil degraded

beyond an acceptable level with increased use. This level was arbitrarily set at between 1.9 and 2.0 volts as judged from the pyrolyser unit voltmeter. Replacement of the pyrolyser coil provided a return to the regular electrical input of 2.25 v and normal pyrolytic patterns.

A University of Alberta Computing services Graphitizer digitizer was used in an attempt to replace manual measurement and recording of retention times and peak heights. The graphitizer records automatically the positions of points outlining a map or strip chart trace when a light emitting diode cursor is passed over the trace converting x-y plots to digital representations on tape. It soon became apparent that the main difficulty using the digitizer was the inability to identify the individual peaks from the digitized data. Variation in peak retention time made it difficult to determine whether some peaks were absent or not based on the digitized data. No attempt was made at digitizing during pyrolysis-chromatographic runs because identification of the individual peaks over all the pyrograms could not be made on the extracted pyrogram data alone.

The internal marker peaks were always off scale because of the difficulty in accurately measuring sufficiently small quantities. Considerable time was spent in preliminary runs to discover the correct attenuation changes necessary to keep all other peaks on scale. Peak heights of the internal

marker peaks were therefore ignored. A logarithmic recorder attenuator would have allowed unattended operation of the gas chromatograph after pyrolysis was initiated, but we did not have this equipment.

Some pyrolysis products of fungal and other samples would condense on the inside wall of the pyrolysis chamber after each run, causing a buildup of tar-like material. If left undisturbed, a noticable increase in base line drift would result. Condensation of pyrolytic products on the chamber wall was thought to be a source of variation in relative peak heights.

Summary of adopted procedures for extracting numeric data from pyrograms

Identification of each peak over all the pyrograms was made visually by superimposing each pyrogram over one produced by strain 854 of Arthroderma tuberculatum. Some samples of each strain were spiked with methyl-caproate and methyl-undecanoate as internal markers. These markers along with other characteristic peaks helped to identify homologous peaks among the various pyrograms by a process of visual 'best-fit' over short regions of the pyrograms. Each peak was assigned a number to correspond with the homologous peak in the reference pyrogram. An illustration of this procedure is given in Fig. 3. For each numbered peak the actual retention distance in millimeters and its height in

chart units were recorded. Missing peaks were assigned a value of zero. Peaks were scored for their heights in chart units as the vertical distance from the mid-point of base width (Wi), as in Fig. 3. This procedure automatically eliminates baseline drift as a factor in the measurement of peak heights. Peaks which were off scale were recorded as maximum (100). Peaks hidden by the presence of the internal markers were scored as no-compare (-1). Peak heights were standardized to compensate for variation in sample size by dividing the height of each peak by the average height of all the peaks in that pyrogram.

Procedures for analysis of data

Recorded data was then entered onto computer disk files. The data from all pyrograms was analysed to find the maximum, minimum, range and mean of the heights for each peak. Table VII shows the variation in peak heights of pyrograms of replicate samples. The variation about the mean was also determined for the peak retention times, as shown in Table IV. The degree of variation in the retention times of the internal markers about their means were then compared with the variations of those peaks preceding the markers in each pyrogram, as in Table V.

A cluster analysis based on average differences of equally-weighted standardized peak heights among the pyrograms (OTU's) was performed as described in Methodology.

Additional cluster analyses on the same data but with altered weighting to take into consideration the range of peak heights and the variation among replicates of each strain were also performed, and compared with the first cluster analysis.

RESULTS AND DISCUSSION

Test of column efficiency and resolution

The efficiency and the resolution of the column as operated in this GLC apparatus, were determined at gas flow rates corresponding to five different pressures. The results are given in Table II. The upper-left quadrant of the table shows the resolution of four pairs of hydrocarbons at five different carrier gas pressures. It can be seen that for this short series, the longer, the chain length the better the resolution. The best resolution for all pairs was obtained at the carrier gas flow rate corresponding to a pressure of 20 psi.

The upper-right quadrant shows the theoretical plate equivalents of the column as determined for benzene at the same pressures. The most efficient operation (relatively narrow peaks) was also obtained at 20 psi. The minimum value for HETP in Table II is similar to those reported by Nikelly (1972) and Blumer (1973). The lower half of the table shows that efficiency and resolution decrease with age and use of the column. Over the six month period covered in the table, approximately 120 pyrograms were run. In the last 2 month period, usage was minimal, and the rate of decline was somewhat less than during the period of heavy use. This indicates that column coat degradation is an important factor in the use of PGLC for taxonomic studies,

even though it is mostly ignored in published accounts. The change in operating character of this column did not appear to be sufficient to affect the identification of homologous peaks.

Qualitative differences in pyrograms

One to five replicate pyrograms were prepared for each of the 21 strains studied to yield (see Table III for number of replicates of each strain) a total of 65 pyrograms. Figs. 7 to 14 show representative pyrograms. Visual comparison of the pyrograms showed 58 different peaks, which were numbered 1 to 58. Peaks 1 and 2 went off scale and their heights were recorded as no-compare (-1). The retention time of peak 11 corresponded to that of ethanol which was used in sample preparation and frequently carried over. Therefore height data obtained from it was ignored in the final analysis. Peaks 26 and 54 were observed when markers were added to the sample and were also scored no-compare for those pyrograms. Peaks 57 and 58 were ignored in calculating proximities because many of the pyrograms were terminated before their elution from the column.

Thirteen of the 56 peaks are absent in some of the pyrograms. Fig. 15 shows some of these peaks present in a pyrogram of Nannizzia gypsea. Table III shows the distribution of these peaks over the strains analysed. A

(+) indicates the peak was present in all of the replicates of that strain. A (0) indicates the peak was absent in all of the replicates. In variable strains, the number of replicates is given for which the peak is present. Peak 3 was present in all replicates of two positive and three negative mating-type strains of Arthroderma tuberculatum and absent from the other ten. In Arthroderma benhamiae this peak was present in all replicates of (-) strains and absent in (+) strains. Peak 5 turned up in only 1 replicate of Arthroderma tuberculatum but was present in both mating types of all other three species. Peak 13 was regularly present in all of the strains studied except 3181 and 3182. Peak 25 discriminates between 1007 and all other Arthroderma tuberculatum strains. It was found in one replicate of both mating mating types of Arthroderma benhamiae and in all replicates of Nannizzia gypsea and Nannizzia incurvata. Peak 31 discriminates Nannizzia gypsea from all other species studied. Peak 35 was absent only in strains 1095-, 1266-, 1450- of Arthroderma tuberculatum and in only one replicate each of 1998- and 3172-. Peak 52 was absent in all strains except in three out of four replicates of Arthroderma benhamiae 2823-. Peak 56 was peculiar to strains of Arthroderma tuberculatum, being absent from the other three species. The only major peak of varaible occurrence was peak 45; the other peaks of variable occurrence were small ones. The presence or absence of some peaks was apparently correlated with mating type in Arthroderma benhamiae and Nannizzia gypsea, but not in

A. tuberculatum. Note that we might have been misled if we had only had one strain of each mating type of A. tuberculatum, for example 1967 (+), and 1998 (-). The difference between the mating types of the other species might also disappear if more strains were studied.

Variation in retention time and use of markers

Table IV shows the range, mean and standard deviation of the raw retention times for each peak over the 65 pyrograms. There is a gradual increase in standard deviation over the first 10 peaks. These peaks were eluted during the initial isothermal portion of the run. Peaks 11 to 43 represent the temperature programed portion. The variation in the standard deviation remains relatively constant from peak 11 to 25. Peak 26 represents the first internal marker, methyl caproate. Variation in standard deviation among peaks 27 to 43 also remains relatively constant. Another gradual increase in the standard deviation coincides with the running of the final isotherm from peaks 44 to 58, including the marker peak 54 (methyl-undecanoate). The amount of variation in retention time of each peak about the mean based on these pyrograms is shown in Table V. In most cases there is a good correlation in retention time variation between the marker peak 26 and the average in retention time for peaks (12-25) preceding and peaks (27-43) following it. The variation in marker peak 54 was generally similar to the average for peaks (41-53).

Table V shows the average differences from mean retention retention times for groups of peaks 12-25, 27-40 and 41-53. The difference from the mean is also shown for the marker peak, 26 and 54. The sign of each difference indicates whether it is to the left (-) or right (+) side of the mean.

Marker correction

A procedure was devised to rescale the raw retention times of certain pyrograms using the drift of the marker peaks. The appropriate correction factor to be applied to a peak with any given raw retention time was read from a graph such as the example shown in Fig. 23. Each retention time value was corrected for variation by the addition or subtraction of the amount indicated by the graph for that pyrogram. This procedure eliminated any overlap in retention time between 44 of the 58 peaks in twelve pyrograms representative of the four species studied. Table VI shows an area of each of the nine pyrograms where some overlap in peak retention between homologous peaks occurred even with the corrected retention time values. However it was possible to identify most individual peaks by their corrected absolute retention times alone, as well as detecting missing peaks.

The method of using markers to correct for variation in retention time works best for those peaks nearest the markers. Although all peaks were identified visually, it

appears that it would have been reasonably satisfactory to automate using the above correction procedure.

Peak heights

Column A in Table VII shows the weighted average within strain range of the peak height for each peak over all the OTU's. Each number in column-A was computed by the following procedure; 1) the range of peak heights over the replicates of each strain was determined, 2) an average was calculated, over all strains, weighting the range for each strain by the number of replicates included. Column-B shows the range of peak height values over all the OTU's and was computed by subtracting the the minimum from the maximum peak height values for each peak. Column-C shows the average within strain range as a % of the range in peak height over all the OTU's. In taking an average we assumed that there was a rough equivalence in the variation for each peak in the different strains. Columns-D (1-3) show the range for each peak for three strains of Arthroderma tuberculatum. It can be seen that, for these strains our assumption was mostly correct. The fact that the average within strain variation is 16 % of the variation found over the different strains is not surprising since PGLC is affected by a large number of variables and also since the strains studied belonged to closely related species.

For some cluster analyses, each peak was weighted to

take into account the average variation in peak height among the replicate pyrograms.

Analysis of proximities

The pyrograms were all quite similar to each other ranging in relative similarity from 95 to 60 % with a median near 77 %. The histogram in Fig. 16 shows the frequency distribution of proximities. It can be seen that the five most similar pairs have a relative distance of 0.05 and 0.06 which corresponds to a similarity of 94-95 %. The nearest neighbor table from the TAXMAP program showed that the nearest neighbor of each OTU was another replicate of the same strain.

Cluster analysis

The clustering of the OTU's resulting from the equally weighted analysis is shown in Table VIII. Table IX shows the clusters from an analysis weighted for the information content of each peak assuming equal intra-strain variation for all peaks. Table X shows an analysis weighted for information content using the average intra-strain range for each peak. The minimum intercluster discontinuities as determined by the computer analysis (see Table XI) were used to construct taxometric maps by triangulation. An approximate configuration for fitting the clusters into a 2-dimensional display was obtained by the MDSCAL program.

Fig. 17 shows the relative MDSCAL positions of the clusters after the equally weighted analysis. A total of 19 clusters appear. Fig. 18 shows the same clusters as positioned by the TAXMAP mapping (The single isolated clusters are not shown in Fig. 18 for simplicity) procedure, which positions each cluster at an undistorted distance from its two nearest neighbors. Eight clusters contain the forty OTU's (pyrograms) belonging to the fifteen strains of Arthroderma tuberculatum. One two-cluster set contains the six OTU's belonging to the two strains of Nannizzia gypsea, the other two-cluster set contains six OTU's belonging to two strains of Arthroderma benhamiae. One cluster contains five OTU's belonging to strains of Nannizzia incurvata.

The remaining six OTU's form single member clusters. These single-OTU clusters were stray replicates of the cluster which contains their nearest neighbor. Fig. 14 shows a pyrogram of an isolated single cluster. The maximum relative distance between the two most distant OTU's in any one cluster of Arthroderma tuberculatum is 0.067, Arthroderma benhamiae 0.057, Nannizzia gypsea 0.046 and for Nannizzia incurvata 0.060. The minimum relative distance between any one cluster of Arthroderma tuberculatum and that of Arthroderma benhamiae is 0.050, Nannizzia gypsea 0.045 and Nannizzia incurvata 0.043. Thus some replicates of the strains of each species were more similar to some replicates of strains from different species than to some of their own

species. For example, some replicates of Nannizzia incurvata and Nannizzia gypsea in clusters 2 and 7 respectively were closer to the most centrally located cluster of Arthroderma tuberculatum, cluster 5, than were some other clusters of A. tuberculatum such as 1 or 4. Nevertheless, the clusters belonging to each species are grouped into contiguous areas of the classification space.

Figures 19 and 20 show the relative positions of the clusters after a weighted analysis, weighted according to the information content of each peak assuming equal intra-strain variation for all peaks. Figures 21 and 22 show the relative positions of the clusters after an analysis weighted for information content using the average intra-strain range for each peak. Some clusters formed in each of the two weighted analyses contains mixtures of species and hence do not agree with the identification of the strains as well as the clusters found in the equally weighted analysis. Although the inclusion of intra-strain variation as a factor in the analysis should improve the classification, it did not do so. We have no explanation for this anomaly.

CONCLUSIONS

Pyrolysis gas-liquid chromatography was suggested by Reiner (1965) as an attractive possibility for the classification of microorganisms because each strain produced its own unique pattern of pyrolytic fragments and the procedure was relatively simple and reproducible. He suggested that the pyrograms were a sort of fingerprint of the organism. Since the pyrogram is produced by degradation of the whole organism, the eluted components should reflect, in some way, the results of the entire metabolism, and hence, hopefully, the activity of the entire genome. It remains to be proven, however that similarity in the proportions of pyrolytic degradation fragments is directly related to closeness of genetic relationship. Seviour et al. (1974) pointed out that pyrolysis obliterates tertiary molecular configurations to produce a relatively limited number of heat stable, or more volatile fragments. The final elution pattern may depend less on the nature of the larger molecular configuration in the organism than on the relative proportions of the simpler moieties in their make-up. Indeed, Seviour et al. (1974) found that pyrograms of Eucalyptus tree roots and of their mycorrhizal fungus were remarkably similar. Thus the range of applicability of PGLC for solving taxonomic problems remains to be demonstrated.

Even before this larger problem can be tackled, it

remains to be shown that the quantitative agreement between replicate samples is good enough to permit the reliable detection of differences between similar organisms. Those workers who have tried to make quantitative comparisons of whole pyrograms and who included replicate samples in their work, have expressed reservations on this point, for example, Carmichael et al. (1973) and Seviour et al. (1974).

In this study, it was confirmed that technical problems are a major barrier to the application of PGLC to taxonomy. The most important limitations found were as follows.

1) Failure to resolve the pyrolysate into individual peaks. It is known from mass-spectral analysis studies by Simmonds (1970) that even the apparent, partially separated peaks may be mixtures. The use of long, capillary columns did not substantially improve the resolution over that previously obtained on the same chromatograph with packed columns, see Carmichael et al. (1973). Because the peaks are overlapped and not separated, the problems resulting from retention time drift, baseline drift and sample size variation are intensified. Seviour et al. (1974) pointed out that uneven shift in the baseline of pyrograms in publications by Cone and Lechowich (1970) and Reiner and Hicks (1972) have been directly associated with changes in relative peak heights.

2) Drift in baseline between pyrograms. Drift in baseline between pyrograms was compensated for by obtaining peak height as the distance from the apex of the peak to the

midpoint of the peak base between its inflection points. Even so some multi-component peaks were difficult to work with because slight variations in relative retention time could also lead to inaccurate peak identification and inaccurate peak height measurements.

3) Variation in sample size. Levy (1967) suggested that variation in sample size caused changes in relative heights of peaks between replicate pyrograms. Variation in sample size between pyrograms caused alterations in the mean heights of peaks. It was also considered to cause the appearance or disappearance of peaks of minor size between replicates. Variations in sample size were compensated for by dividing each peak height by the mean height of all peaks in the same pyrogram.

4) Variation in retention time. Variations in retention times of peaks between pyrograms are considered the result of variations in temperature program or gas flow rates, and are a potential source of error in the identification of homologous peaks. Misidentification can lead to erroneous reports of qualitative and quantitative differences between pyrograms. A rigid adherence to an arbitrary criterion for distinguishing peaks by retention time can lead to erroneous reports of similarity between pyrograms. In this study the use of known internal marker compounds which volatilized as components of known retention times aided in the identification of homologous peaks by superimposing one pyrogram over another. By using the variation of each marker about its mean and applying it as a

correction factor to the retention times of neighboring peaks, it was found that most peaks could be identified by their corrected retention time alone, a system suited for automation.

5) The pyrolysis of fungal samples led to the accumulation of tars in the pyrolysis chamber, and the accumulation of high molecular weight components in the column, which interferes with the normal elution patterns of samples towards the high temperature end of the program. The tars that accumulated in the pyrolysis chamber were removed by using cotton swabs, and occasionally washing the chamber with soap and water.

6) Column coat degradation. A decrease in column efficiency and resolution was noticed over a period of six months column operation and was most noticeable after two to three months of heavy use. Column coat degradation is considered an important factor in the use of PGLC for taxonomic studies and is mostly ignored in published accounts.

7) Aging of the pyrolysis coil. Aging of the nickel-chrome pyrolysis coil appeared to cause noticeable variations in the elution patterns of replicate pyrograms. The replacement of used coils with new ones temporarily solved the problem.

8) The use of capillary columns of 0.25 mm internal diameter required larger than usual sample sizes to be pyrolysed along with the use of gas-sample splitters.

Visual comparison of the pyrograms allowed some strains to be identified by the presence or absence of particular peaks. In some cases the mating types could be distinguished.

On quantitative comparison, most but not all, replicates were similar enough to be clustered together. Some strains belonging to the same species were also similar enough to be grouped in one cluster. Other strains of a single species differed sufficiently to be put in separate clusters. The nearest neighbor to each OTU (pyrogram) was always a replicate pyrogram of the same strain. The 15 strains of A. tuberculatum were distributed among 8 clusters. The two strains of A. benhamiae were placed into 3 clusters, those of N. gypsea into two and N. incurvata into one.

The clustering of all strains is roughly the same in the equal and both log weighted analyses. However the log weighted analysis grouped together into one cluster strains of A. tuberculatum, N. gypsea and N. incurvata indicating the similarity of the major fractions of the pyrograms belonging to these strains.

The intra-cluster cluster discontinuity in all maps was large compared to the between cluster variation. That is the maximum distances within clusters are of similar size to the distance between clusters. The distances between all

clusters are roughly the same, and clusters of A. tuberculatum are roughly the same distance from each other as they are from other species. Nonetheless the equally weighted map does show a separation of genera into different parts of the classification space. Although the within cluster distance between the two most distant OTU's in some clusters was occasionally bigger than the between cluster distances, this does not invalidate the classification, because the most distant OTU's within clusters are bridged by intermediate OTU's while the relative discontinuity between clusters is empty.

PGLC still appears to be a potentially valuable source of taxonomic data, but further investigations are necessary to determine the most appropriate equipment and procedures. In addition the nature of PGLC requires that meticulous attention be given to the control and standardization of all aspects of the technique if any useful results are to be obtained.

TABLE I

SOURCES OF FUNGI USED FOR PYROLYSIS-GLC

UAMH No.	Name	Mating type	source
854	Arthroderma tuberculatum	-	N. M. McClung, Atlanta, as Sepedonium from soil, rec'd 1960.
1007	" "	+	D. Frey, Inst. Med. Res. N. S. W. as No.18b, from soil, rec'd 1961.
1095	" "	+	NRRL as A-10, 054, rec'd 1961.
1266	" "	-	N. M. McClung, Univ. Georgia, as No.25 from soil, rec'd 1960.
1450	" "	-	N. M. McClung, Univ. Georgia, as No. R.53, rec'd 1962.
1724	" "	-	J. W. Carmichael, Univ. Alta., as ?, from soil, 1963.
1905	" "	+	G. F. Orr, UTAH, as RSA 1254, from ?, rec'd 1964.
1967	" "	+	E. Varsavsky, Argentina, as EV7A, from soil, rec'd 1964.
1998	" "	-	G. F. Orr, UTAH, as 0-919, from soil, rec'd 1964.
2831	" "	+	A. Padhye, India, as ?, from soil, rec'd 1967.
3147	" "	+	G. F. Orr, UCLA, as No.2503, from human isol., 1969..
3181	" "	+	CMI as No.86177, from feathers, rec'd 1969.
3182	" "	-	CMI as No.86178, from feathers, rec'd 1969.
3172	" "	-	P. V. Kurup, Ohio State Univ., as No.8L57, from soil, rec'd 1969.
3229	" "	?	J. W. Carmichael, Calif., as No.53-1-a, from soil, rec'd 1969.
2822	Arthroderma benhamiae	+	NCDC as X-797, rec'd 1967.
2823	" "	-	NCDC as X-798, rec'd 1967.

TABLE I cont'd

1485	Nannizzia gypsea	+ CMI as 86175 single ascospore isolate from 80558, rec'd 1962.
1486	" "	- CMI AS 86176 single ascospore isolate from 80558, rec'd 1962.
2835	Nannizzia incurvata	- CMI as 86518, rec'd 1968.
2936	" "	+ CMI as 86523, rec'd 1968.

TABLE II

COLUMN EFFICIENCY AND RESOLUTION

Carrier gas, psi	Resolution C^5-C^6	C^6-C^7	C^7-C^8	C^8-C^9	n Benzene	HETP(mm)
15	4.64	6.80	10.10	16.70	30,815	1.67
20	4.80	7.20	11.10	18.50	34,698	1.44
25	4.60	7.00	11.20	16.50	33,060	1.51
35	4.60	6.10	9.40	12.60	23,716	2.11
45	4.10	5.20	7.00	10.20	------	----
	Effect of Age					
	Month					
25	0	4.60	7.00	11.20	16.50	33,060	1.51
25	2	4.80	6.80	10.10	15.60	29,234	1.71
25	4	4.70	6.20	9.80	13.50	25,938	1.92
25	6	4.60	6.20	8.50	13.90	22,773	2.19

GLC Column : 50 m x 0.25 mm i. d.
Chart speed : 0.75 inches/minute
Column coat : Carbowax-20M, 8%.
Resolution units as defined in Methodology

TABLE III

QUALITATIVE DIFFERENCES IN SELECTED PEAKS

Strain	Total Replicates	Peak Number 3	5	13	25	31	35	36	37	41	45	50	52	56
A. tuberculatum														
854-	4	0	0	+	0	0	+	0	+	+	0	+	0	0
1007+	2	0	0	+	+	0	+	0	+	+	0	1	0	0
1095+	4	0	0	+	0	0	0	0	+	3	0	0	0	0
1266-	3	+	0	+	0	0	0	0	+	+	0	1	0	0
1450-	2	0	1	+	0	0	0	0	+	+	0	+	0	+
1724-	2	0	0	+	0	0	+	+	+	0	0	0	0	0
1905+	4	0	0	+	0	0	+	1	3	0	0	0	0	+
1967+	2	0	0	+	0	0	+	+	+	+	0	0	0	1
1998-	4	+	0	+	0	0	3	+	+	3	+	1	0	0
2831+	4	+	0	1	0	0	+	+	+	+	+	0	0	0
3147+	3	0	0	1	0	0	+	+	+	+	+	0	0	1
3172-	3	0	0	2	0	0	2	+	0	+	+	2	0	0
3229?	3	0	0	+	0	0	+	+	0	1	+	0	0	0
3181+	1	+	0	0	0	0	+	0	+	+	+	1	0	0
3182-	3	+	0	0	0	0	+	0	+	+	+	0	0	0
A. benhamiae														
2822+	5	0	2	+	1	0	+	+	0	+	+	4	0	0
2823-	4	+	2	1	1	0	+	+	0	2	+	+	3	0
N. gypsea														
1485+	4	3	3	+	+	+	+	0	+	3	+	0	0	0
1486-	3	+	+	+	+	+	+	2	+	2	+	+	0	0
N. incurvata														
2935-	3	+	1	2	+	0	+	0	+	+	+	1	0	0
2936+	2	+	1	1	+	0	+	0	+	+	+	1	0	0

Units = number of positive replicates
+ = all replicates positive

TABLE IV

VARIATION IN PEAK RETENTION TIME
DATA FROM 65 PYROGRAMS

Peak no.	Maximum	Minimun	Range	Mean	Standard deviation
1	9.0	8.0	1.0	8.46	2.07
2	10.5	9.2	1.3	9.69	2.66
3	11.5	10.0	1.5	10.61	3.86
4	12.7	11.2	1.5	12.09	2.54
5	15.5	13.7	1.8	14.52	5.07
6	17.0	15.2	1.8	16.19	3.63
7	18.7	16.7	2.0	17.89	4.28
8	23.5	20.5	3.0	22.32	5.96
9	27.5	24.5	3.0	26.07	6.40
10	30.0	26.2	3.8	28.20	7.72
11	32.5	28.5	4.0	30.55	8.46
12	36.0	31.5	4.5	33.59	9.12
13	40.7	35.5	5.2	38.45	10.27
14	44.0	39.7	4.3	41.74	9.16
15	47.7	42.5	5.2	44.49	9.94
16	51.0	45.2	5.8	48.58	9.99
17	53.0	47.5	5.5	50.68	10.57
18	57.5	53.0	4.5	55.34	8.74
19	60.7	56.5	4.2	58.67	9.44
20	62.5	58.7	3.8	60.73	8.53
21	64.7	60.0	4.7	62.52	8.63
22	67.0	63.0	4.0	64.74	8.31
23	68.5	64.7	3.8	66.30	9.51
24	70.0	65.7	4.3	67.65	10.83
25	71.5	68.0	3.5	69.73	11.12
26	75.0	71.0	4.0	72.28	9.68
27	74.5	70.5	4.0	71.78	9.46
28	77.0	72.5	4.5	74.20	11.73
29	79.5	74.5	5.0	76.29	11.10
30	81.2	76.7	4.5	78.25	11.03
31	82.5	79.5	4.5	80.05	9.89
32	84.0	77.2	6.8	80.54	11.29
33	85.5	80.0	5.5	81.97	10.75
34	87.5	83.0	4.5	84.66	10.29
35	90.0	85.5	4.5	87.21	10.16
36	94.2	90.2	4.0	91.51	6.31
37	96.5	90.5	6.0	92.94	12.69
38	99.2	91.7	7.5	95.85	12.63
39	100.5	95.0	5.5	96.98	11.64
40	103.0	98.2	4.8	99.79	10.96
41	105.0	100.5	4.5	101.97	10.80
42	106.5	102.0	4.5	103.40	10.57
43	108.5	103.5	5.0	105.02	11.03
44	112.0	107.0	5.0	108.53	10.11
45	113.0	108.5	4.5	109.66	9.88
46	115.5	110.0	5.5	111.72	10.61
47	117.0	111.5	5.5	113.24	10.81

TABLE IV cont'd

Peak no.	Maximum	Minimun	Range	Mean	Standard deviation
48	120.0	113.7	6.3	115.76	11.76
49	121.5	115.5	6.0	117.39	11.56
50	123.5	117.0	6.5	119.22	12.47
51	124.0	119.0	5.0	120.95	10.94
52	125.2	120.0	5.2	122.55	12.12
53	127.0	121.0	6.0	123.59	12.23
54	129.2	122.5	6.7	124.81	14.50
55	130.0	121.5	8.5	126.67	14.01
56	135.5	127.0	8.5	130.62	15.44

Time in chart units (0.10 inches). 1 unit = 24 secs.

TABLE V

AVERAGE OF DIFFERENCES FROM MEAN RETENTION TIMES IN EACH GROUP

Peaks 12-25	26	27-40	41-53	54	Peaks 12-25	26	27-40	41-53	54
-10	-7	-11	-17	-25	+11	+9	+7	+4	+1
+4	0	-5	-8	-13	+4	+4	+2	+6	+16
-5	-7	-9	-14	-21	-11	-12	-12	-15	-21
-10	-7	-11	-12	-18	-2	+3	+2	-1	-11
+3	+5	-1	+1	+2	-8	-7	-5	-7	-18
-14	-16	-6	-11	-21	+18	+13	+8	+17	+19
-1	-2	-6	-7	-8	-5	-3	-5	+1	+1
0	-2	-1	-4	+1	+1	-3	-1	0	+1
+25	+27	+23	+27	+26	-1	-2	0	0	+1
-1	+2	0	0	+1	0	-2	-4	-3	-1
-3	0	-3	-2	-1	+1	+1	0	-1	-3
+1	0	0	0	-1	-5	-7	-5	-5	-8
+3	+2	+1	+3	+3	-2	-6	-4	-3	-3
-4	-7	-1	+1	+3	-17	-12	-6	-4	-6
-5	-7	-4	-4	+1	0	+2	0	-1	+1
0	-2	+3	+3	+6	+17	+19	+17	+18	+19
-6	-7	-5	-4	-6	+14	+24	+23	+36	+30
+11	+12	+17	+18	+26					

Time in chart units (0.10 inches). 1 unit = 24 secs.
Each line = one pyrogram

TABLE VI

CORRECTED RETENTION TIMES

OTU's Peak	1	2	3	4	5	6	7	8	9	M
28	765	766	755	768	768	754	769	768	763	762
29	0	0	0	0	0	802	0	781	772	783
30	805	804	799	807	806	812	802	807	0	808
31	820	817	811	822	819	827	816	821	822	820
32	846	848	841	847	849	847	843	851	842	847
33	872	873	866	873	0	872	870	877	867	872
34	0	0	0	0	0	914	0	0	0	915
35	826	924	914	927	927	929	919	915	914	929
36	960	957	951	960	960	962	960	963	936	958
37	970	968	970	969	971	974	973	974	947	970
38	998	997	990	999	998	1001	997	999	993	998
39	1023	1018	1004	1019	0	1016	1017	1025	1013	1020
40	1040	1035	1021	1037	1037	1038	1030	1039	1033	1034
41	1054	1052	1043	1053	1053	1051	1050	1053	1048	1050
42	1090	1090	1073	1090	1090	1086	1084	1093	1083	1085
43	0	0	0	0	0	1093	1093	0	0	1097
44	1121	1120	1112	1122	1121	1118	1118	1124	1115	1117

Time in chart units (0.10 inches). 1 unit = 24 secs.

TABLE VII

VARIATION IN PEAK HEIGHTS OF PYROGRAMS OF REPLICATE SAMPLES

Peak	A Weighted Average Within Strain Range	B Range over All OTU's	C Variation as a % of Range	D Within Strain Range for Strains I	II	III
3	0.34	5.77	5.89	0	0	0
4	1.45	7.70	18.30	1.60	1.68	1.26
5	0.21	3.02	6.95	0	0	0
6	0.34	1.34	25.37	0.13	0.14	0.44
7	1.15	4.67	24.62	1.43	1.00	2.09
8	0.50	1.96	25.51	0.33	0.22	0.55
9	0.23	1.08	21.29	0.23	0.04	0.12
10	0.26	2.14	12.14	0.15	0.42	0.10
12	0.37	2.43	15.22	0.23	0.08	0.25
13	0.20	1.83	10.92	0.15	0.03	0.08
14	0.17	0.86	19.76	0.02	0.23	0.11
15	0.62	3.41	18.18	0.60	0.36	0.30
16	0.19	0.83	20.89	0.17	0.20	0.19
17	0.49	7.44	6.58	1.90	0.62	0.93
18	1.55	1.97	78.68	0.36	0.49	0.12
19	0.06	0.27	22.00	0.18	0.03	0.06
20	0.07	0.33	21.23	0.03	0.06	0.08
21	0.09	0.50	18.00	0.07	0.06	0.05
22	0.24	1.19	20.16	0.12	0.11	0.18
23	0.10	0.69	14.49	0.03	0.06	0.05
24	0.11	0.53	20.75	0.37	0.03	0.12
25	0.03	0.55	5.54	0.07	0.03	0.00
27	0.08	2.26	3.53	0.05	0.06	0.03
28	0.11	1.22	9.01	0.10	0.22	0.06
29	0.37	1.67	22.15	0.40	0.01	0.08
30	0.30	1.53	19.60	0.50	0.13	0.10
31	0.02	0.22	9.09	0	0	0
32	0.15	1.67	22.38	0.23	0.36	0.12
33	0.18	0.92	19.56	0.10	0.06	0.06
34	0.26	1.37	18.97	0.40	0.08	0.16
35	0.06	0.36	16.60	0.10	0.06	0
36	0.10	0.62	16.10	0	0	0
37	0.10	0.73	13.69	0.13	0.09	0.18
38	0.17	0.83	20.48	0.14	0.03	0.12
39	0.17	0.68	25.00	0.17	0.36	0.17
40	0.19	0.69	27.53	0.18	0.03	0.04
41	0.11	0.62	17.74	0.06	0.03	0.15
42	0.16	1.12	14.28	0.11	0.03	0.04
43	0.32	1.86	17.20	0.04	0.05	0.40
44	1.18	5.75	20.52	0.45	0.35	1.40
45	0.55	6.08	9.04	0	0	0
46	0.51	3.70	13.78	0.70	0.12	0.28
47	0.21	1.17	17.94	0.34	0.06	0.09

TABLE VII cont'd

Peak	A Weighted Average Within Strain Range	B Range over All OTU's	C Variation as a % of Range	D Within Strain Range for Strains I	II	III
48	0.50	3.57	14.00	0.71	0.04	0.71
49	0.19	0.90	21.00	0.10	0.04	0.20
50	0.10	0.57	17.54	0.06	0.06	0
51	0.28	2.94	9.52	0.12	0.04	0.22
52	0.04	0.68	5.88	0	0	0
53	0.38	1.57	24.20	0.85	0.08	0.37
55	0.17	2.44	6.96	0.32	0.06	0.04
56	0.02	0.32	6.25	0	0	0

Height as a fraction of the average height of all peaks.

TABLE VIII

RESULTS OF COMPUTER ANALYSIS OF PYROGRAM DATA

DATA EQUALLY WEIGHTED

Cluster No.	OTU Nos	Dist Best Link	OTU Best Link	AvgOf New Links	DropIn Avg	Far OTU	Dist Far OTU	Flag	OTU Name
1	8								A. tu1095+
	9	0.05							A. tu1095+
	10	0.06	8	0.062	0.011	9	0.07		A. tu1095+
	7	0.07	8	0.080	0.018	10	0.10		A. tu1095+
	5	0.12	8	0.132	0.052	10	0.14		A. tu1007+
	6	0.07	5	0.132	0.000	9	0.16		A. tu1007+
	3	0.10	5	0.123	0.009	9	0.14		A. tu0854-
	1	0.06	3	0.127	0.005	10	0.16		A. tu0854-
	2	0.07	3	0.131	0.003	10	0.17		A. tu0854-
	4	0.12	2	0.151	0.020	9	0.19		A. tu0854-
	11	0.15	8	0.173	0.022	4	0.21	1000	A. tu1266-
2	62								N. in2935-
	63	0.06							N. in2935-
	61	0.10	63	0.110	0.051	62	0.12		N. in2935-
	65	0.10	62	0.122	0.012	61	0.16		N. im2936+
	64	0.12	63	0.140	0.018	65	0.17		N. in2936+
	44	0.14	63	0.170	0.030	61	0.18	1000	A. tu3182-
3	16								A. tu1724-
	17	0.06							A. tu1724-
	18	0.13	16	0.134	0.074	17	0.14		A. tu1905+
	19	0.08	18	0.127	0.006	17	0.16		A. tu1905+
	20	0.08	19	0.128	0.001	17	0.17		A. tu1905+
	21	0.12	20	0.151	0.023	18	0.17		A. tu1905+
	43	0.13	19	0.173	0.022	17	0.19	1000	A. tu3182-
4	12								A. tu1266-
	13	0.06							A. tu1266-
	11	0.07	12	0.071	0.010	13	0.08		A. tu1266-
	14	0.15	12	0.161	0.089	11	0.17		A. tu1450-
	15	0.14	14	0.166	0.006	13	0.18		A. tu1450-
	8	0.15	11	0.174	0.008	15	0.22	4	A. tu1095-

TABLE VIII cont'd

Cluster No.	OTU Nos	Dist Best Link	OTU Best Link	AvgOf New Links	DropIn Avg	Far OTU	Dist Far OTU	Flag	OTU Name
5	41								A. tu3181+
	44	0.07							A. tu3182-
	42	0.12	44	0.136	0.064	41	0.15		A. tu3182-
	43	0.12	42	0.136	0.000	41	0.15		A. tu3182-
	35	0.13	42	0.157	0.021	41	0.19		A. tu3172-
	36	0.08	35	0.141	0.016	41	0.19		A. tu3172-
	37	0.10	35	0.164	0.023	41	0.22		A. tu3172-
6	29								A. tu2831+
	30	0.08							A. tu2831+
	28	0.08	29	0.082	0.002	30	0.08		A. tu2831+
	31	0.09	30	0.118	0.036	29	0.14		A. tu2831+
	25	0.12	31	0.160	0.041	29	0.19		A. tu1998-
	26	0.09	25	0.139	0.021	28	0.17		A. tu1998-
	24	0.11	25	0.141	1.002	29	0.18		A. tu1998-
	27	0.12	26	0.166	0.025	28	0.21	1000	A. tu1998-
7	55								N. gy1485+
	57	0.09							N. gy1485+
	56	0.10	55	0.104	0.018	57	0.11		N. gy1485+
	54	0.12	56	0.123	0.019	57	0.13		N. gy1485+
	44	0.14	54	0.170	0.047	55	0.18	1004	A. tu3182-
8	59								N. gy1486-
	60	0.09							N. gy1486-
	63	0.15	60	0.168	0.077	59	0.18	1004	N. in2935-
9	47								A. be2822+
	48	0.10							A. be2822+
	46	0.11	47	0.115	0.015	48	0.12		A. be2822+
	45	0.11	46	0.141	0.026	48	0.16		A. be2822+
	49	0.13	47	0.173	0.032	45	0.20	1000	A. be2822+
10	38								A. tu3229?
	39	0.12							A. tu3229?
	35	0.15	39	0.183	0.066	38	0.22	1004	A. tu3172-
11	50								A. be2823-
	51	0.12							A. be2823-
	53	0.16	51	0.175	0.054	50	0.19	1000	A. be2823-

12	32								A. tu3147+
	34	0.13							A. tu3147+
	36	0.14	32	0.162	0.030	34	0.18	4	A. tu3172-
13	22								A. tu1967+
	23	0.14							A. tu1967+
	20	0.14	22	0.151	0.014	23	0.16	4	A. tu1905+
14	52								A. be2823-
	53	0.15							A. be2823-
	49	0.16	53	0.170	0.022	52	0.19	1000	A. be2822+

Isolated OTU's (single member clusters).

15	27	A. tu1998-
16	33	A. tu3137+
17	37	A. tu3172-
18	49	A. be2822+
19	58	N. gy1486-

Flag 1000 = Drop in average exceeded
0004 = point already member of another cluster

A. tu = Arthroderma tuberculatum
A. be = Arthroderma benhamiae
N. gy = Nannizzia gypsea
N. in = Nannizzia incurvata

TABLE IX

RESULTS OF COMPUTER ANALYSIS OF PYROGRAM DATA

DATA LOG WEIGHTED

Cluster no.	OTU Nos	Dist Best Link	OTU Best Link	AvgOf New Links	DropIn Avg	Far OTU	Dist Far OTU	Flag	OTU Name
1	1								A. tu0854-
	3	0.05							A. tu0854-
	2	0.06	3	0.063	0.013	1	0.06		A. tu0854-
	5	0.09	3	0.105	0.042	1	0.11		A. tu1007+
	6	0.08	5	0.110	0.004	2	0.13		A. tu1007+
	7	0.10	5	0.121	0.012	2	0.14		A. tu1095+
	8	0.06	7	0.110	0.011	2	0.14		A. tu1095+
	9	0.05	8	0.112	0.001	2	0.15		A. tu1095+
	10	0.07	9	0.117	0.006	2	0.17		A. tu1095+
	4	0.11	3	0.139	0.022	9	0.17		A. tu0854-
	20	0.13	3	0.162	0.023	7	0.20	1000	A. tu1905+
2	16								A. tu1724-
	17	0.06							A. tu1724-
	18	0.11	16	0.117	0.059	17	0.12		A. tu1905+
	19	0.10	18	0.123	0.006	17	0.14		A. tu1905+
	20	0.09	19	0.125	0.002	17	0.16		A. tu1905+
	21	0.11	20	0.151	0.026	16	0.17		A. tu1905+
	3	0.13	20	0.162	0.011	17	0.20	4	A. tu0854-
3	62								N. in2935-
	63	0.06							N. in2935-
	65	0.09	62	0.094	0.034	63	0.10		N. in2936+
	61	0.11	63	0.125	0.031	65	0.15		N. in2935-
	54	0.14	63	0.149	0.024	61	0.16		N. gy1485+
	55	0.09	54	0.142	0.007	61	0.17		N. gy1485+
	57	0.09	55	0.144	0.002	61	0.18		N. gy1485+
	56	0.10	55	0.135	0.009	61	0.17		N. gy1485+
	44	0.12	54	0.147	0.012	61	0.17		A. tu3182-
	41	0.08	44	0.162	0.015	61	0.21		A. tu3181+
	43	0.11	44	0.146	0.015	62	0.18		A. tu3182-
	42	0.12	44	0.160	0.013	65	0.19		A. tu3182-
	35	0.13	43	0.186	0.027	61	0.23	1000	A. tu3172-
4	11								A. tu1266-
	12	0.06							A. tu1266-
	13	0.07	12	0.072	0.012	11	0.07		A. tu1266-
	14	0.15	12	0.163	0.091	13	0.17	1000	A. tu1450-

TABLE IX cont'd

Cluster No.	OTU Nos	Dist Best Link	OTU Best Link	AvgOf New Links	DropIn Avg	Far OTU	Dist Far OTU	Flag	OTU Name
5	59								N. gy1486-
	60	0.07							N. gy1486-
	63	0.14	60	0.151	0.082	59	0.16	4	N. in2935-
6	35								A. tu3172-
	36	0.08							A. tu3172-
	37	0.11	35	0.115	0.039	36	0.12		A. tu3172-
	43	0.13	35	0.145	0.030	37	0.17	4	A. tu3182-
7	28								A. tu2831+
	29	0.08							A. tu2831+
	30	0.09	28	0.091	0.013	29	0.09		A. tu2831+
	31	0.11	30	0.140	0.049	29	0.17		A. tu2831+
	25	0.16	31	0.202	0.062	29	0.24	1000	A. tu1998-
8	25								A. tu1998-
	26	0.09							A. tu1998-
	24	0.12	25	0.124	0.030	26	0.13		A. tu1998-
	27	0.14	26	0.160	0.036	24	0.18	1000	A. tu1998-
9	45								A. be2822+
	46	0.10							A. be2822+
	47	0.10	46	0.118	0.020	45	0.14		A. be2822+
	48	0.11	47	0.125	0.007	45	0.16		A. be2822+
	49	0.13	47	0.169	0.044	45	0.19	1000	A. be2822+
10	38								A. tu3229?
	39	0.11							A. tu3229?
	5	0.14	39	0.159	0.052	38	0.18	1004	A. tu1007+
11	22								A. tu1967+
	23	0.12							A. tu1967+
	20	0.15	23	0.146	0.026	22	0.15	4	A. tu1905+
12	52								A. be2823-
	53	0.12							A. be2823-
	51	0.15	53	0.162	0.039	52	0.17	1000	A. be2823-

13	32								A. tu3147+
	34	0.13							A. tu3147+
	36	0.15	32	0.174	0.046	34	0.20	1004	A. tu3172-
14	50								A. be2823-
	51	0.14							A. be2823-
	53	0.15	51	0.179	0.036	50	0.20	1004	A. be2823-
15	14								A. tu1450-
	15	0.15							A. tu1450-
	12	0.15	14	0.163	0.016	15	0.17	4	A. tu1266-

Isolated OTU's (single member clusters).

16	27	A. tu1998-
17	33	A. tu3147+
18	40	A. tu3229?
19	49	A. be2822+
20	58	N. gy1486-
21	64	N. in2936+

A. tu = *Arthroderma tuberculatum*
A. be = *Arthroderma benhamiae*
N. gy = *Nannizzia gypsea*
N. in = *Nannizzia incurvata*

TABLE X

RESULTS OF COMPUTER ANALYSIS OF PYROGRAM DATA

LOG WEIGHTED DATA WITH ALTERED 95% C.I.

Cluster no.	OTU Nos	Dist Best Link	OTU Best Link	AvgOf New Links	DropIn Avg	Far OTU	Dist Far OTU	Flag	OTU Name
1	8								A. tu1095+
	9	0.05							A. tu1095+
	10	0.05	8	0.057	0.010	9	0.06		A. tu1095+
	7	0.06	8	0.071	0.014	10	0.09		A. tu1095+
	5	0.11	8	0.117	0.046	10	0.12		A. tu1007+
	6	0.07	5	0.117	0.000	9	0.14		A. tu1007+
	3	0.09	5	0.111	0.006	9	0.12		A. tu0854-
	1	0.05	3	0.119	0.008	10	0.14		A. tu0854-
	2	0.06	1	0.117	0.002	10	0.15		A. tu0854-
	4	0.10	2	0.131	0.014	9	0.16		A. tu0854-
	11	0.14	8	0.166	0.035	4	0.20	1000	A. tu1266-
2	16								A. tu1724-
	17	0.05							A. tu1724-
	18	0.13	16	0.132	0.078	17	0.13		A. tu1905+
	19	0.08	18	0.120	0.012	16	0.14		A. tu1905+
	20	0.08	19	0.122	0.002	17	0.15		A. tu1905+
	21	0.12	20	0.145	0.024	18	0.17		A. tu1905+
	35	0.14	17	0.162	0.017	18	0.19	1000	A. tu3172-
3	12								A. tu1266-
	13	0.06							A. tu1266-
	11	0.06	12	0.068	0.010	13	0.07		A. tu1266-
	8	0.14	11	0.146	0.079	12	0.15	4	A. tu1095+
4	62								N. in2935-
	63	0.06							N. in2935-
	65	0.08	62	0.087	0.025	63	0.09		N. in2936+
	61	0.09	63	0.111	0.024	65	0.13		N. in2935-
	64	0.12	61	0.143	0.032	65	0.16		N. in2936+
	44	0.13	63	0.153	0.009	64	0.17		A. tu3182-
	41	0.07	44	0.163	0.011	64	0.20		A. tu3181+
	42	0.11	44	0.152	0.011	64	0.18		A. tu3182-
	43	0.11	42	0.142	0.010	62	0.17		A. tu3182-
	54	0.12	44	0.144	0.001	64	0.17		N. gy1485+
	56	0.11	54	0.170	0.026	64	0.21	1000	N. gy1485+

TABLE X cont'd

Cluster No.	OTU Nos	Dist Best Link	OTU Best Link	AvgOf New Links	DropIn Avg	Far OTU	Dist Far OTU	Flag	OTU Name
5	35								A. tu3172-
	36	0.08							A. tu3172-
	37	0.10	35	0.104	0.029	36	0.11		A. tu3172-
	43	0.12	35	0.140	0.036	37	0.16	4	A. tu3182-
6	28								A. tu2831+
	29	0.08							A. tu2831+
	30	0.08	28	0.079	0.001	29	0.08		A. tu2831+
	31	0.09	30	0.116	0.037	29	0.14		A. tu2831+
	25	0.12	31	0.161	0.044	29	0.19	1000	A. tu1998-
7	55								N. gy1485+
	57	0.09							N. gy1485+
	56	0.10	55	0.097	0.011	57	0.10		N. gy1485+
	54	0.11	56	0.117	0.020	57	0.13	4	N. gy1485+
8	25								A. tu1998-
	26	0.09							A. tu1998-
	24	0.11	25	0.109	0.022	26	0.11		A. tu1998-
	27	0.12	26	0.131	0.023	24	0.14		A. tu1998-
	31	0.12	25	0.135	0.003	27	0.16		A. tu2831+
9	59								N. gy1486-
	60	0.09							N. gy1486-
	63	0.14	60	0.151	0.062	59	0.16	4	N. in2935-
10	47								A. be2822+
	48	0.10							A. be2822+
	49	0.10	47	0.125	0.028	48	0.15		A. be2822+
	46	0.10	47	0.127	0.002	49	0.16		A. be2822+
	45	0.12	46	0.164	0.037	49	0.20	1000	A. be2822+
11	38	0							A. tu3229?
	39	0.11							A. tu3229?
	35	0.14	39	0.172	0.062	38	0.20	1004	A. tu3172-
12	50								A. be2823-
	51	0.12							A. be2823-
	52	0.15	50	0.159	0.041	51	0.17	1000	A. be2823-
13	14								A. tu1450-
	15	0.13							A. tu1450-

	12	0.15	14	0.163	0.033	15	0.18	1004	A. tu1266-
14	32								A. tu3147+
	34	0.13							A. tu3147+
	36	0.13	32	0.161	0.028	34	0.19	1004	A. tu3172-
15	52								A. be2823-
	53	0.14							A. be2823-
	50	0.15	52	0.165	0.026	53	0.18	1004	A. be2823-
16	22								A. tu1967+
	23	0.14							A. tu1967+
	36	0.14	23	0.167	0.025	22	0.19	1004	A. tu3172-

Isolated OTU's (single member clusters).

17	33	A. tu3147+
18	40	A. tu3229?
19	45	A. be2822+
20	58	N. gy1486-

A. tu = Arthroderma tuberculatum
A. be = Arthroderma benhamiae
N. gy = Nannizzia gypsea
N. in = Nannizzia incurvata

TABLE XI

CLUSTER DISTANCES
EQUALLY WEIGHTED ANALYSIS

Computer print out of the half matrices from the equally weighted cluster analysis of TAXMAP. The half matrix at the top contains the center-to-center distances of the cluster circles, multiplied by a 1000 to allow printing without a decimal point.
The center-to-center distance is calculated as half the distance between the two most distant OTU's in each cluster (i.e. the radius) plus the nearest neighbor distance between the two clusters. For example the center-to-center distance between cluster 7 and 9 is given in the table as 243 (i.e. 0.243x1000).
The half matrix at the bottom contains nearest neighbor distances between clusters, for example the distance between the nearest neighbors in cluster 7 and 9 is 149.

```
  1
313   2
257 281   3
334 323 337   4
286 277 303 277   5
369 341 299 364 316   6
351 372 362 257 330 327   7
339 363 328 329 287 266 362   8
352 381 349 279 323 387 243 349   9
315 319 350 321 262 354 352 317 345  10
293 311 343 320 249 383 337 337 318 306  11
346 358 325 371 309 322 399 313 382 329 337  12
331 321 249 392 349 384 423 371 428 368 380 359  13
318 320 326 305 255 343 362 324 374 312 317 345 355  14
367 365 338 330 295 318 350 316 349 285 327 278 384 346  15
330 294 300 341 268 336 375 331 357 323 301 350 341 294 334  16
295 277 274 285 212 259 335 267 338 290 284 299 345 207 306 253  17
314 303 335 363 276 364 388 359 344 328 224 352 388 365 356 312 319  18
251 289 289 314 230 359 309 308 304 200 251 307 325 291 327 293 302 283  19
383 379 364 299 306 360 227 338 208 315 340 376 432 352 318 337 310 347 295  20
```

MINIMUM INTER-CLUSTER DISCONTINUITIES.

```
  1
149   2
140 160   3
156 142 202   4
151 138 211 124   5
217 185 191 195 190   6
222 239 270 110 227 277   7
187 207 229 160 161 124 242   8
227 253 267 137 224 271 149 253   9
154 154 232 142 126 201 222 165 219  10
158 172 251 168 140 257 233 211 219 169  11
208 215 230 215 196 192 291 183 279 189 224  12
186 172 148 229 230 247 309 235 318 222 260 235  13
171 169 223 141 134 204 247 186 263 164 196 220 223  14
217 211 232 163 171 177 231 175 235 134 203 150 249 210  15
179 139 192 172 142 194 255 189 241 171 175 220 204 156 193  16
215 193 232 187 158 187 286 195 293 208 230 241 279 140 236 182  17
234 219 298 265 222 292 339 288 299 247 170 293 323 298 287 241 319  18
171 205 252 216 176 287 260 237 260 119 196 248 260 224 257 222 302 283  19
303 295 328 201 252 308 178 267 164 234 286 317 367 285 249 266 310 347 295  20
```

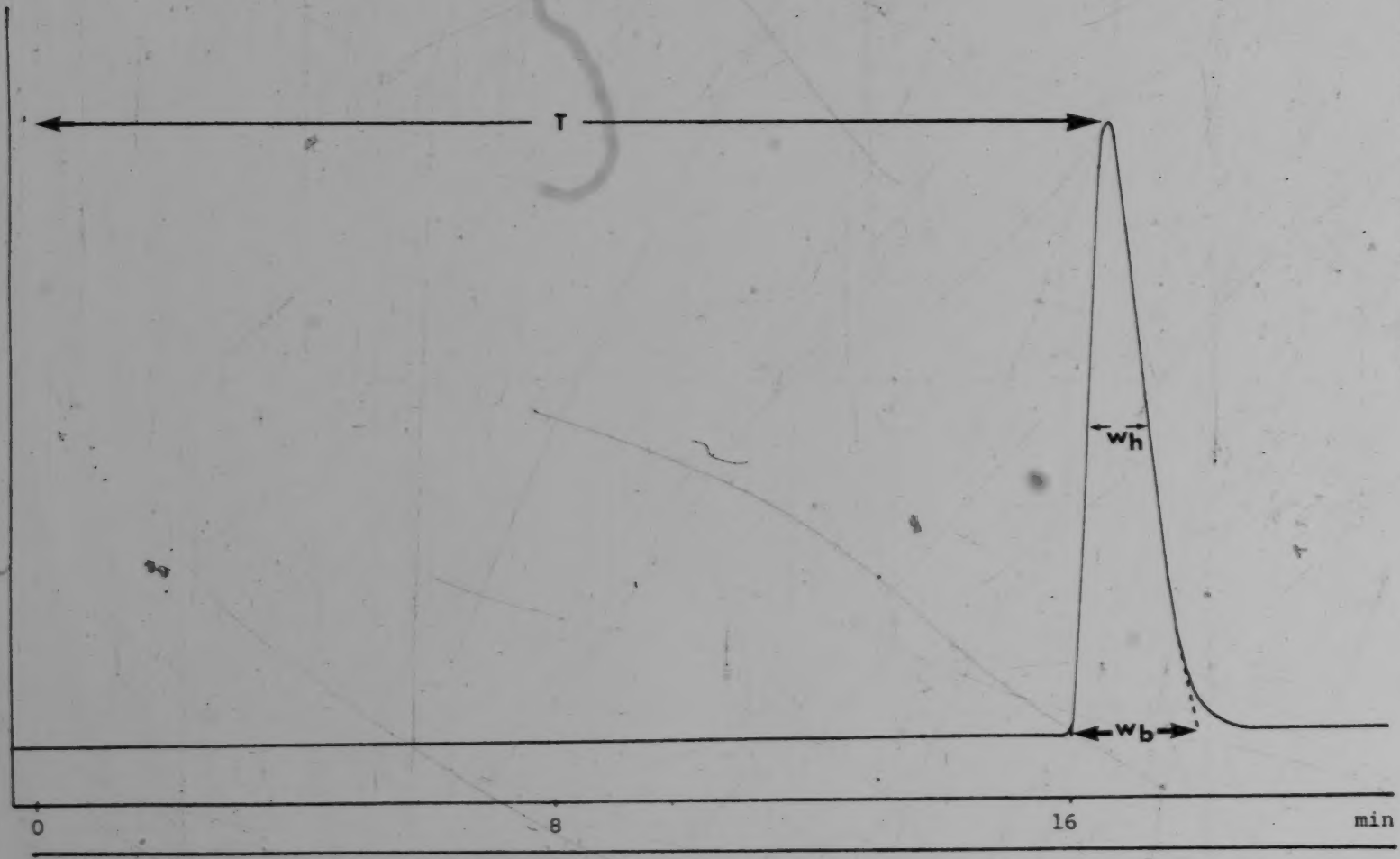


Fig. 1. Chromatogram of benzene. The retention time is shown as (T) in millimeters. The peak width is shown as Wh at half-peak height, or Wb at the base of the peak in millimeters.

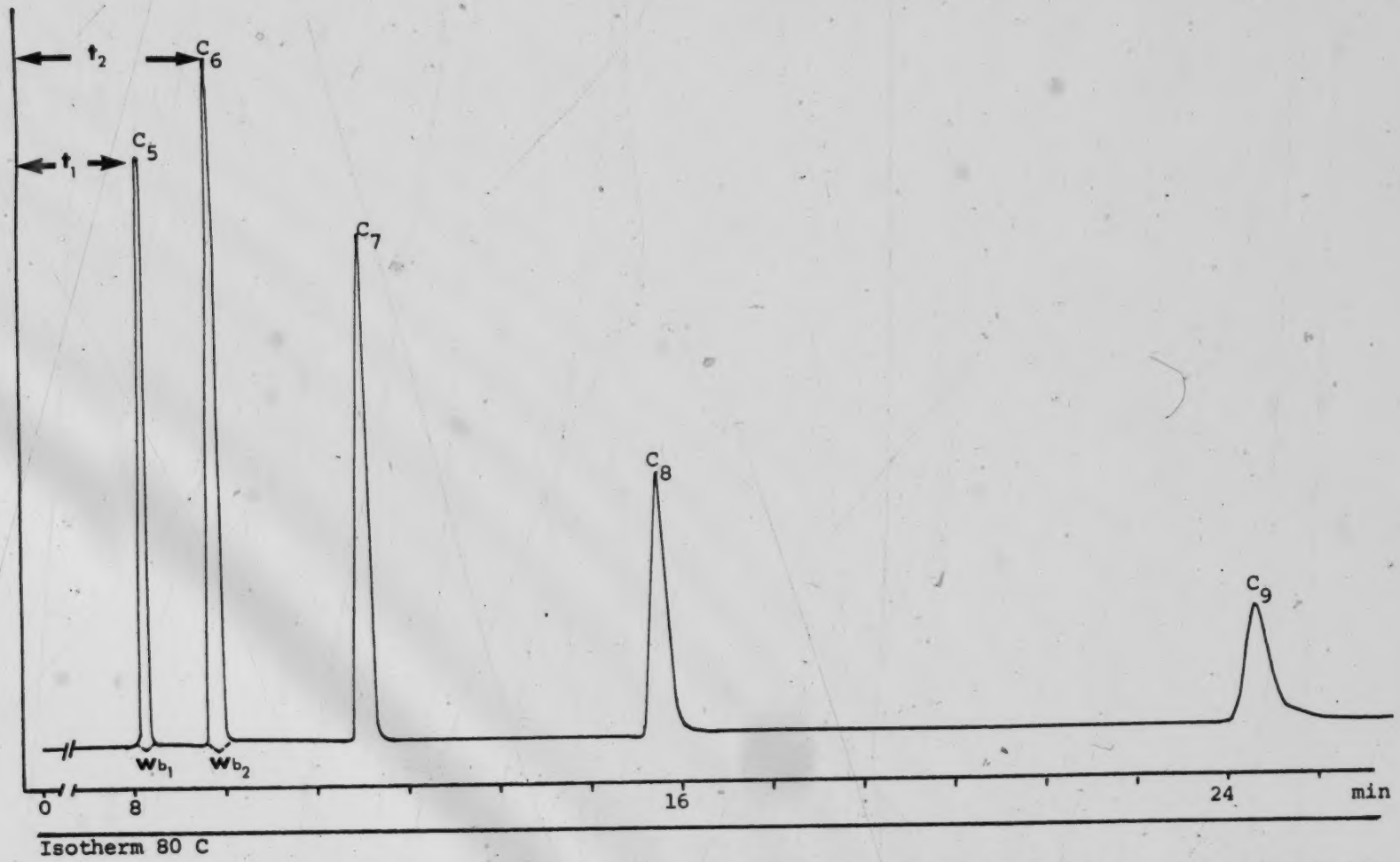


Fig. 2. Carbon-five to Carbon-nine homologous hydrocarbons chromatogram. The retention time of peak one and peak two is (t_1) and (t_2) in millimeters respectively. The peak widths of peak one and two in millimeters is Wb_1 and Wb_2 respectively.

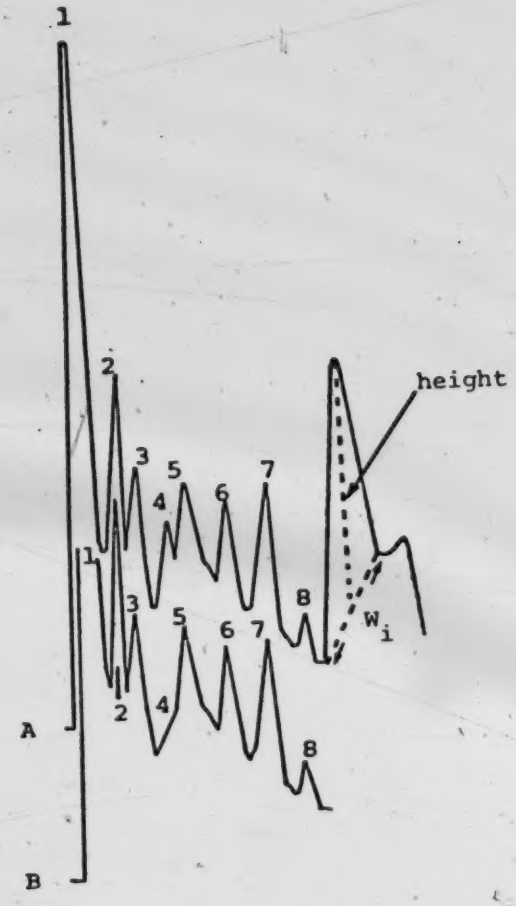


Fig. 3. Partial profile of fungal pyrograms illustrating method of component peak identification. Note that the missing peak in profile B was recorded as peak 4 rather than peak 5 even though the peak recorded as 5 is about mid-way between peaks 4 and 5 in the reference pyrogram A. This was done because the neighboring peaks 3, 6 and 7 are also shifted somewhat to the left.
The peak at the right shows the method of measuring peak height using W_i.

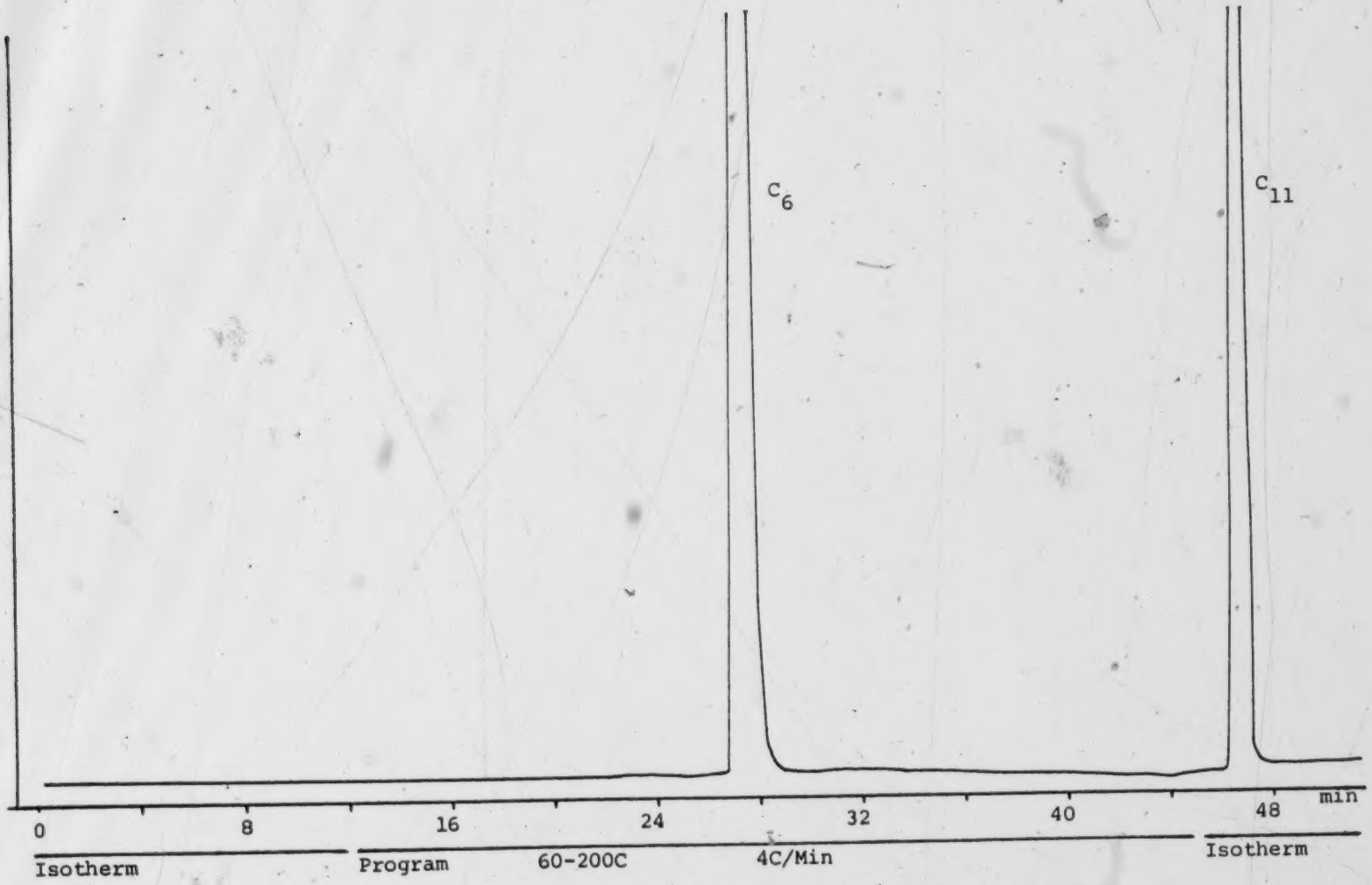


Fig. 4. Pyrogram of Carbon-6 (methyl-caproate) and Carbon-11 (methyl-undecanoate).

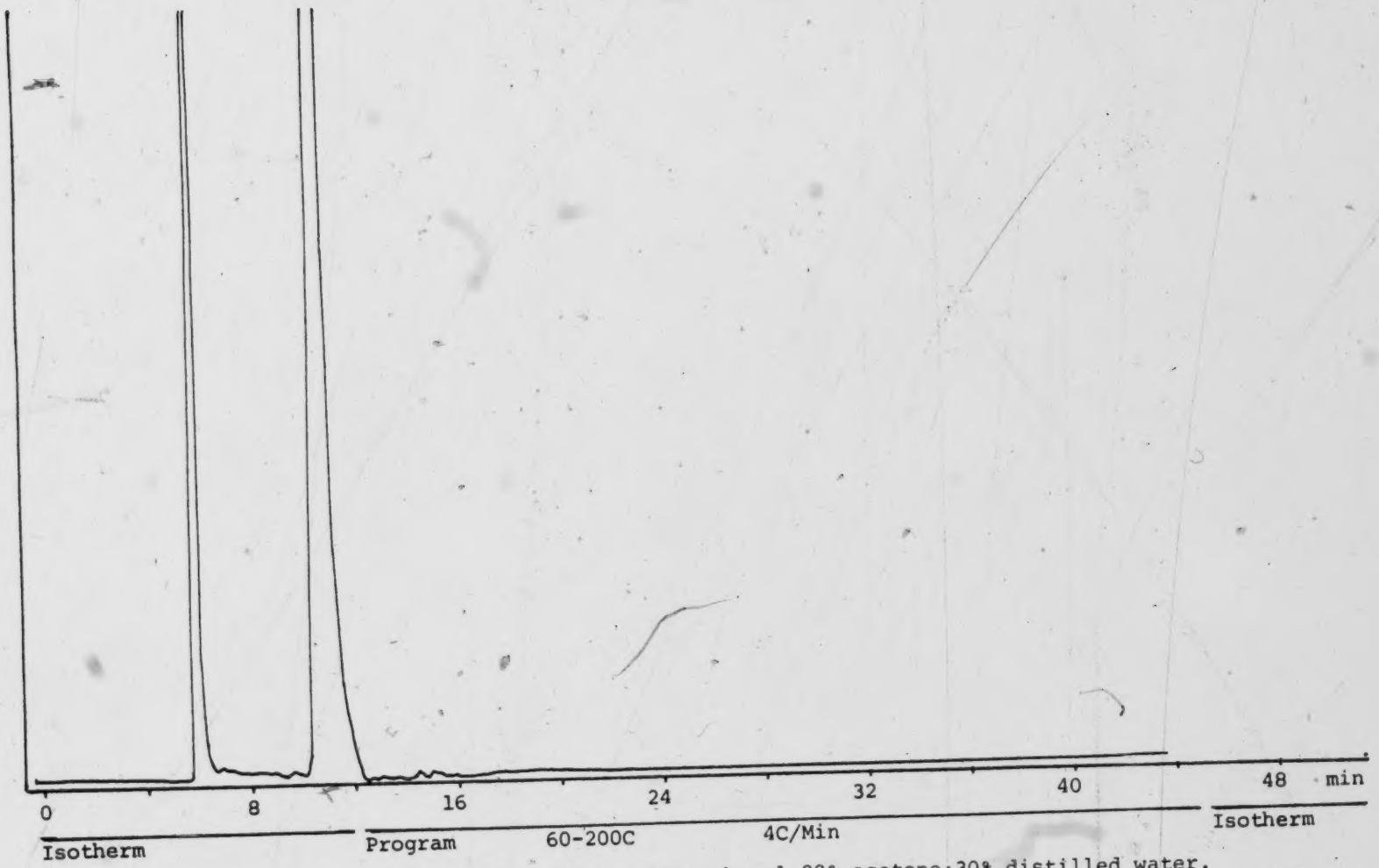


Fig. 5. Pyrogram of sample solvent containing 50% ethanol:20% acetone:30% distilled water.

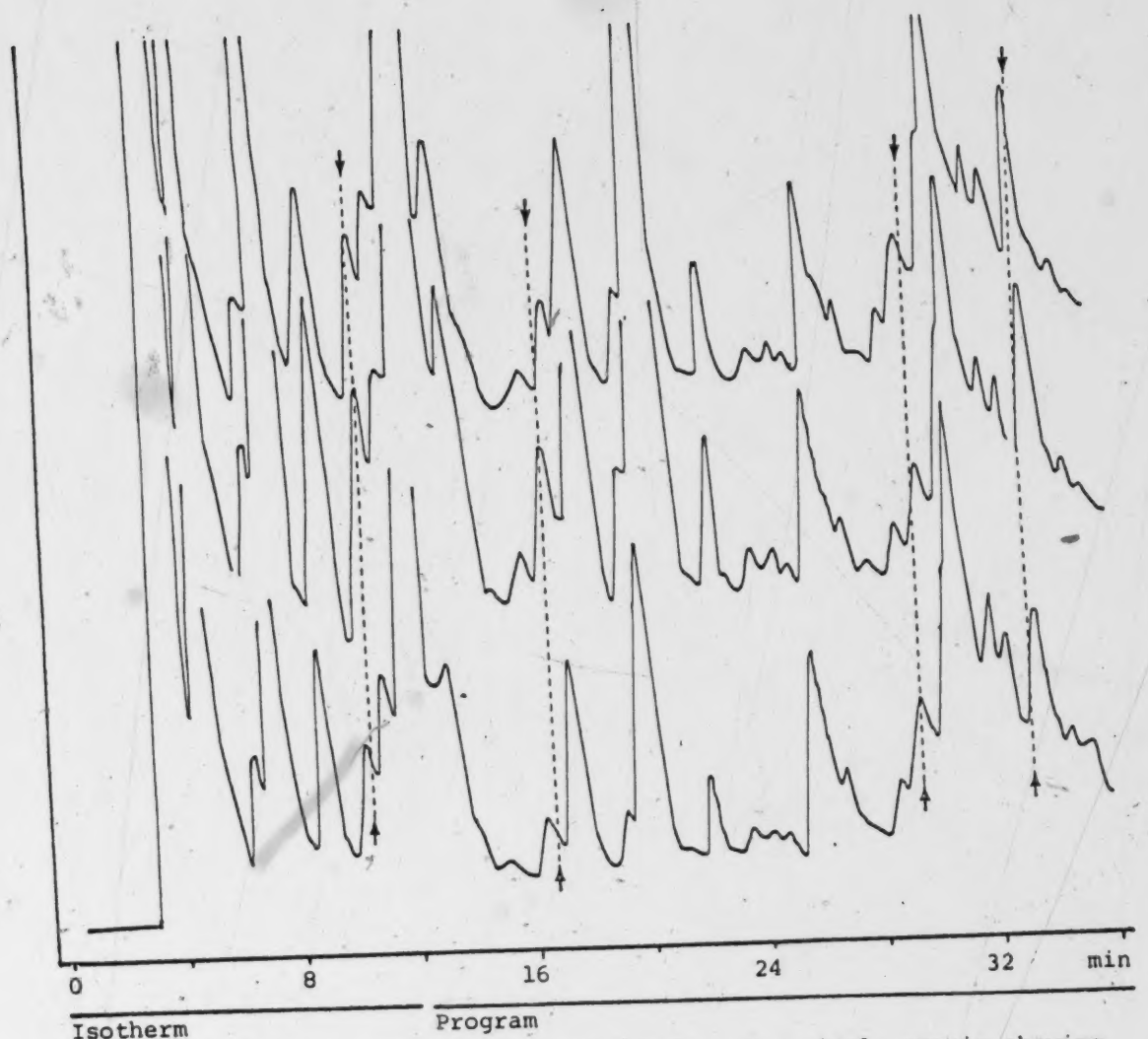


Fig. 6. Pyrograms of three replicate samples of a single strain showing variation in retention time.

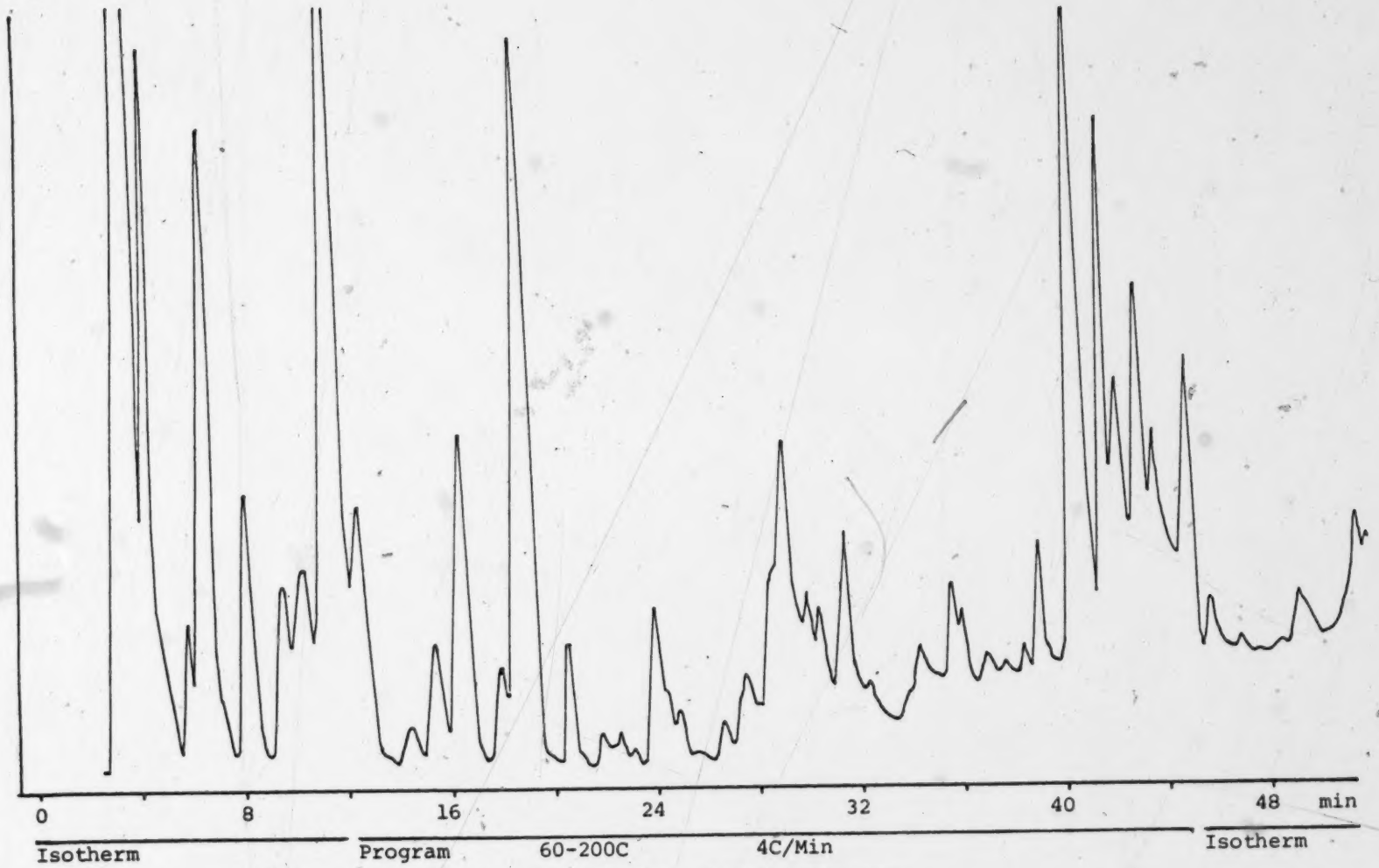


Fig. 7. Pyrogram of Arthroderma tuberculatum 854 (-) replicate one.

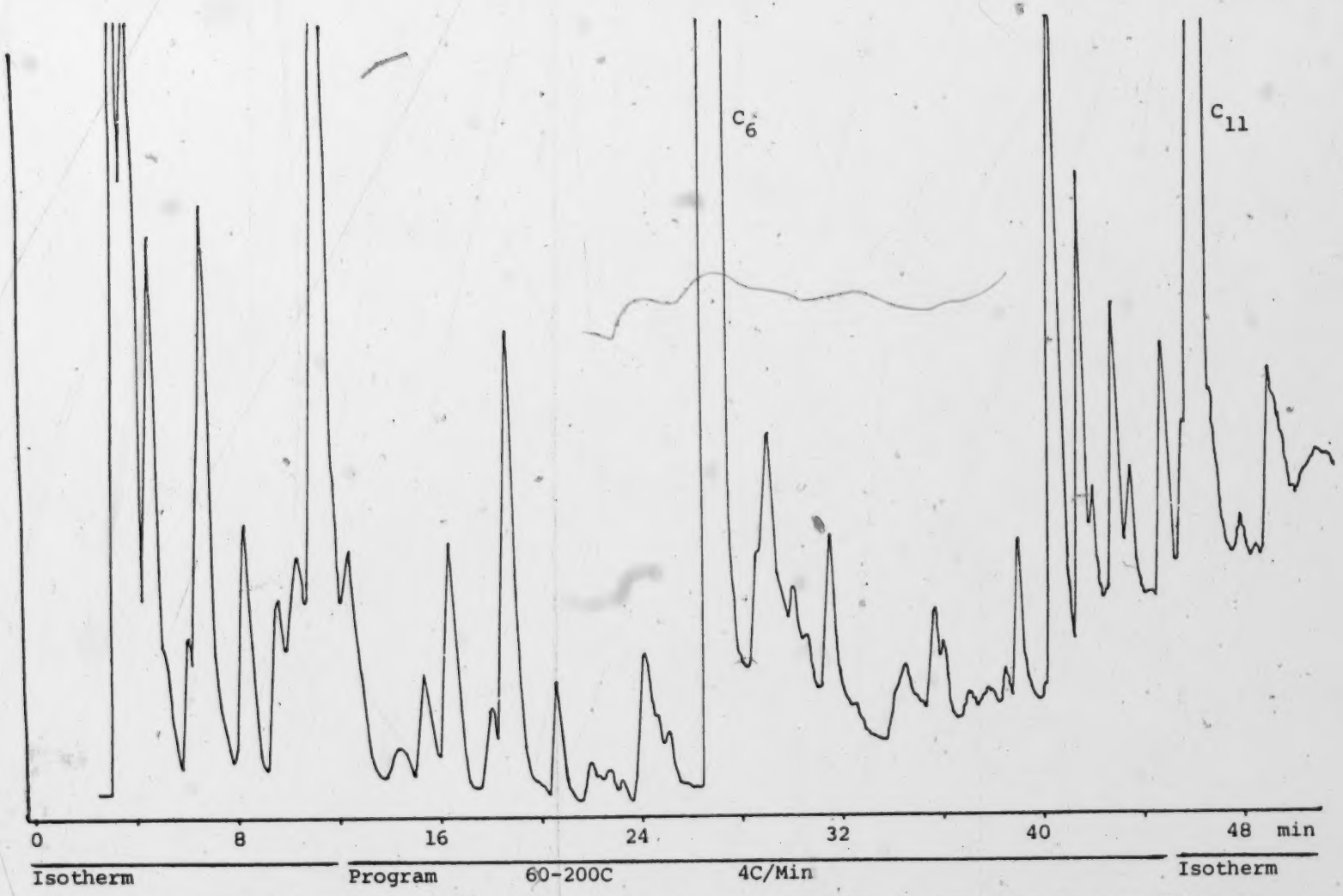


Fig. 8. Pyrogram of Arthroderma tuberculatum 854 (-) replicate two. Internal markers as shown are carbon-6 (methyl-caproate) and carbon-11 (methyl-undecanoate).

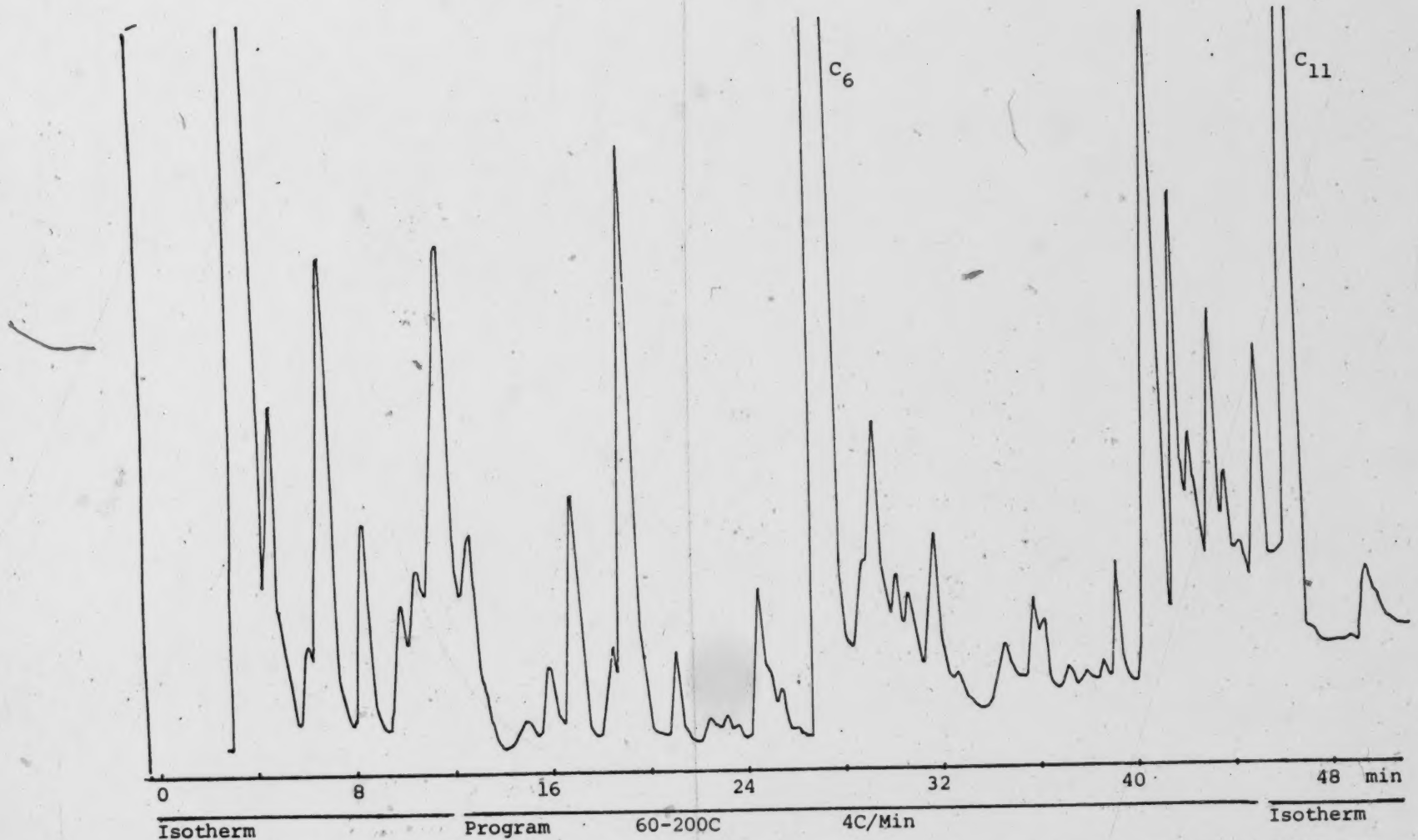


Fig. 9. Pyrogram of Arthroderma tuberculatum 854 (-) replicate three. Internal markers as shown are carbon-6 (methyl-caproate) and carbon-11 (methyl-undecanoate).

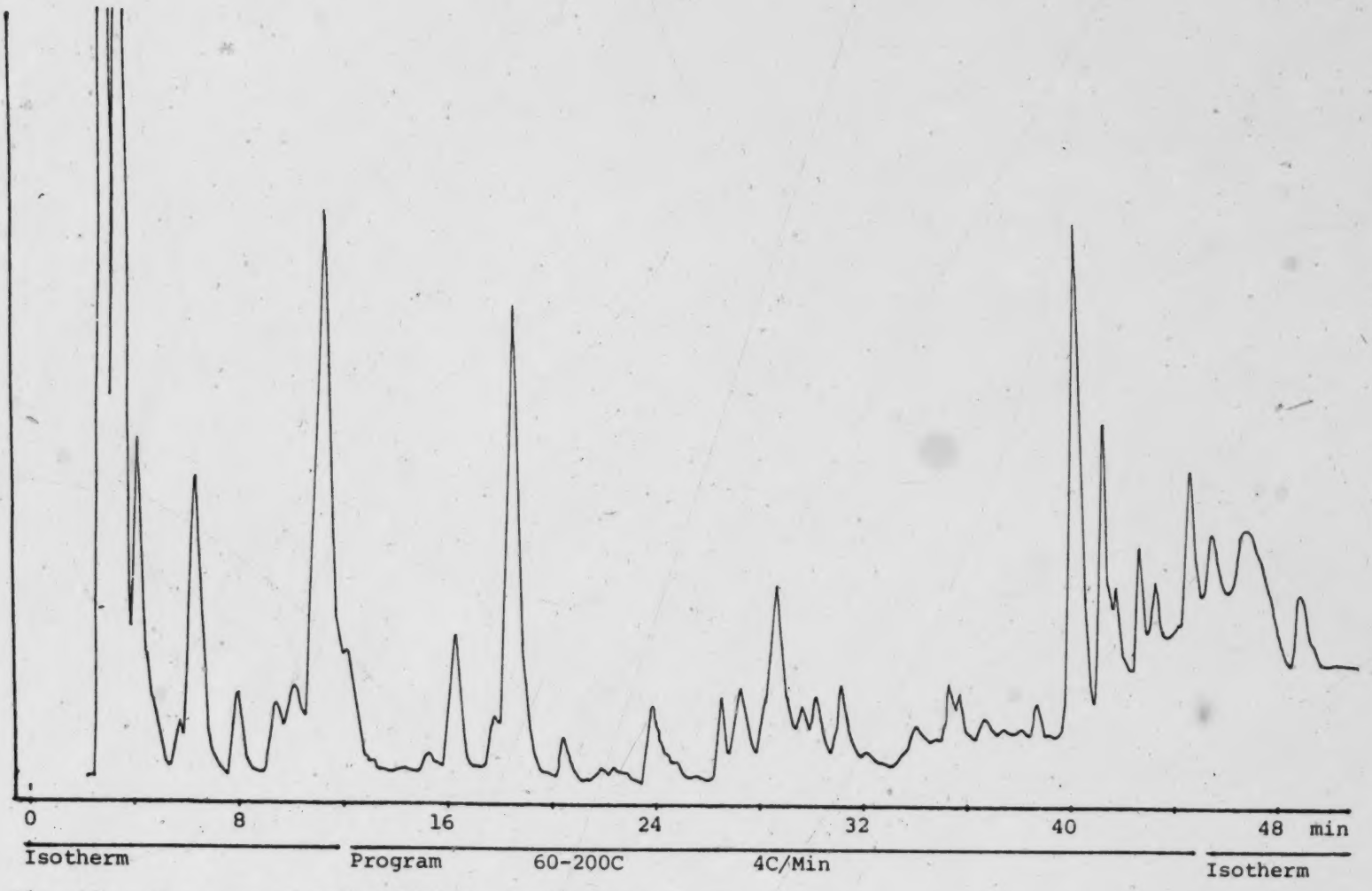


Fig. 10. Pyrogram of Arthroderma benhamiae 2822 (+).

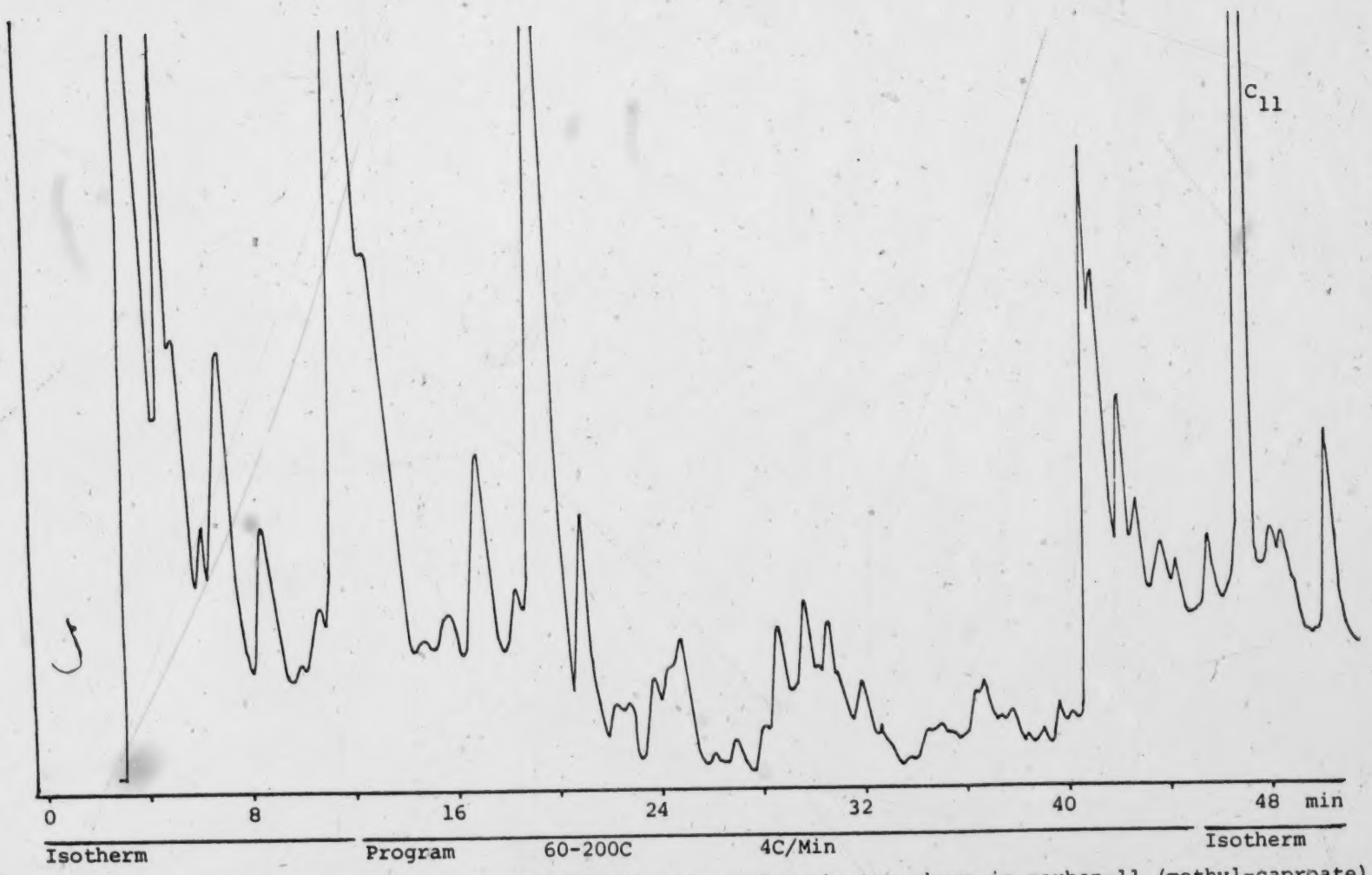


Fig. 11. Pyrogram of Nannizzia gypsea 1485 (+). Internal marker as shown is carbon-11 (methyl-caproate).

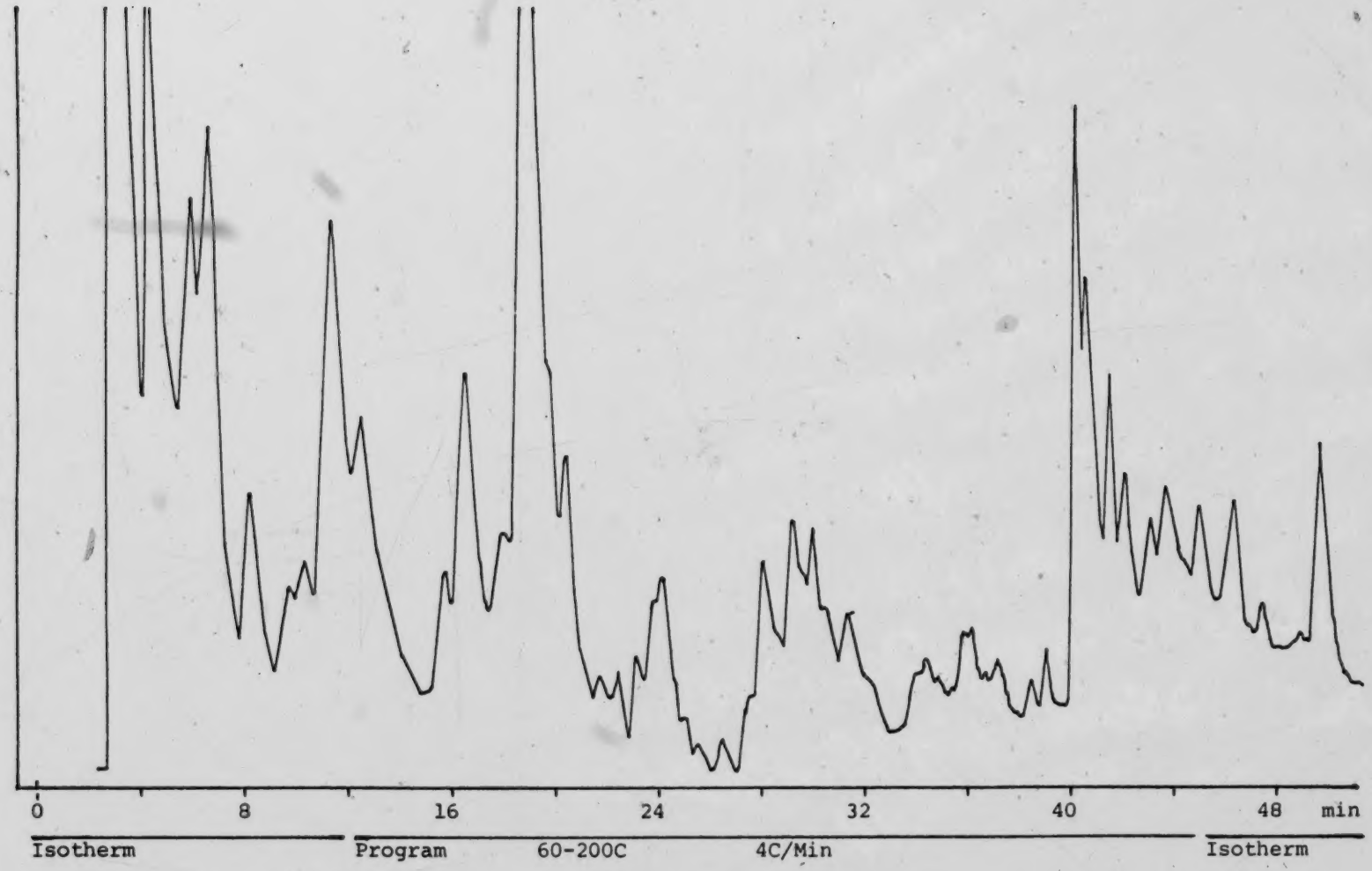


Fig. 12. Pyrogram of Nannizzia incurvata 2936 (+).

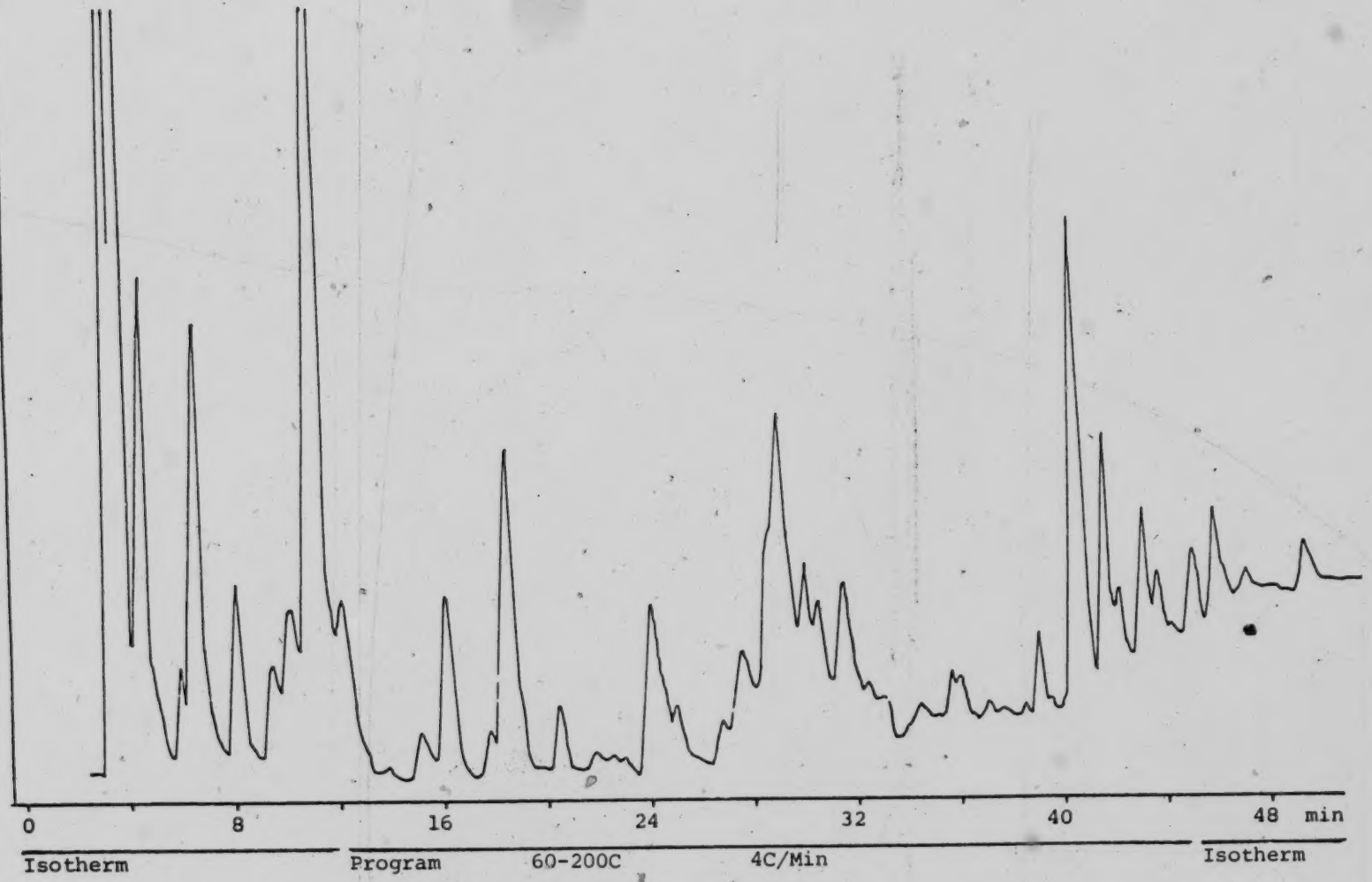


Fig. 13. Pyrogram of Arthroderma tuberculatum 3147 (+).

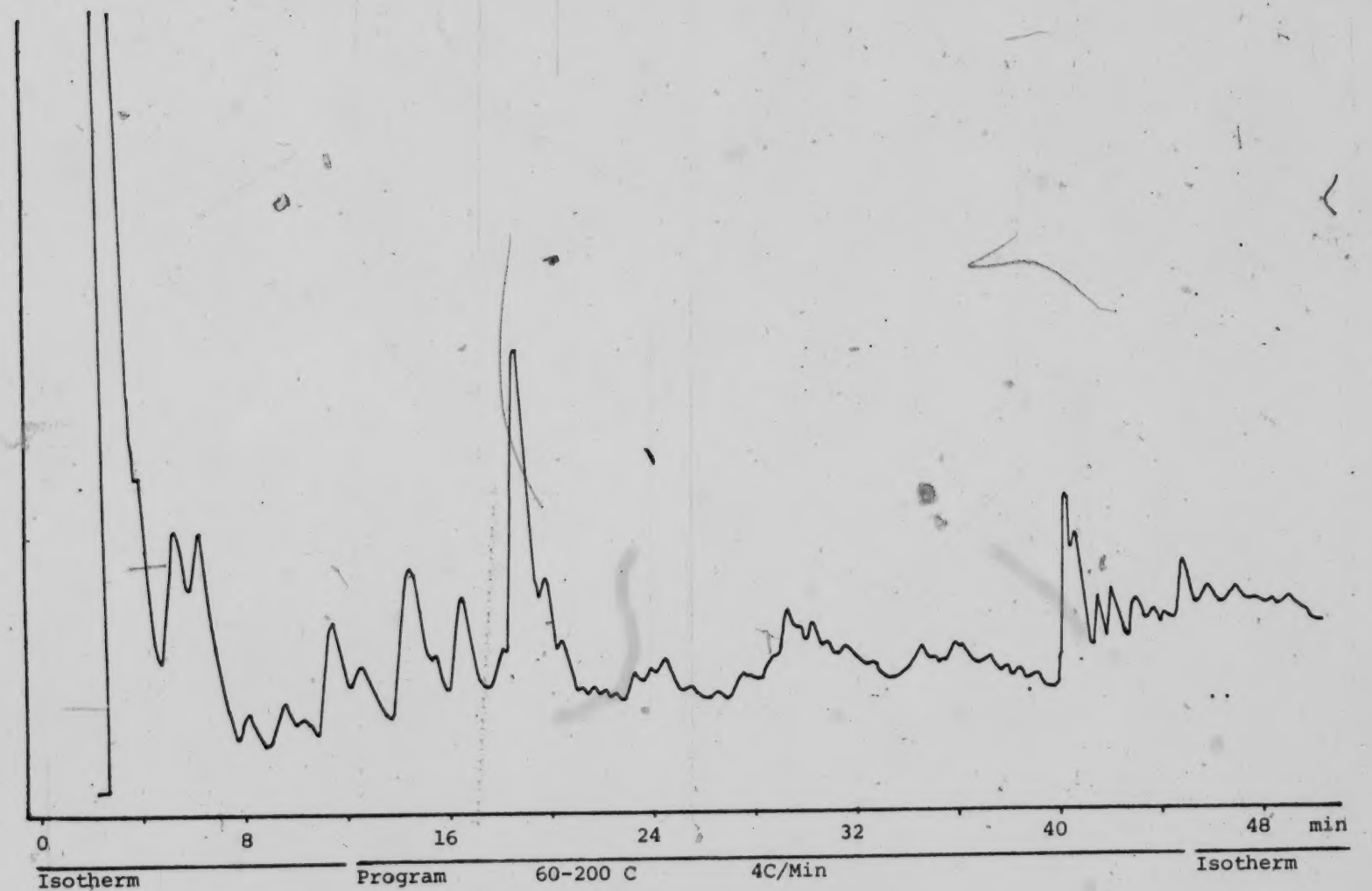


Fig. 14. Pyrogram of Arthroderma tuberculatum 3147 (+).

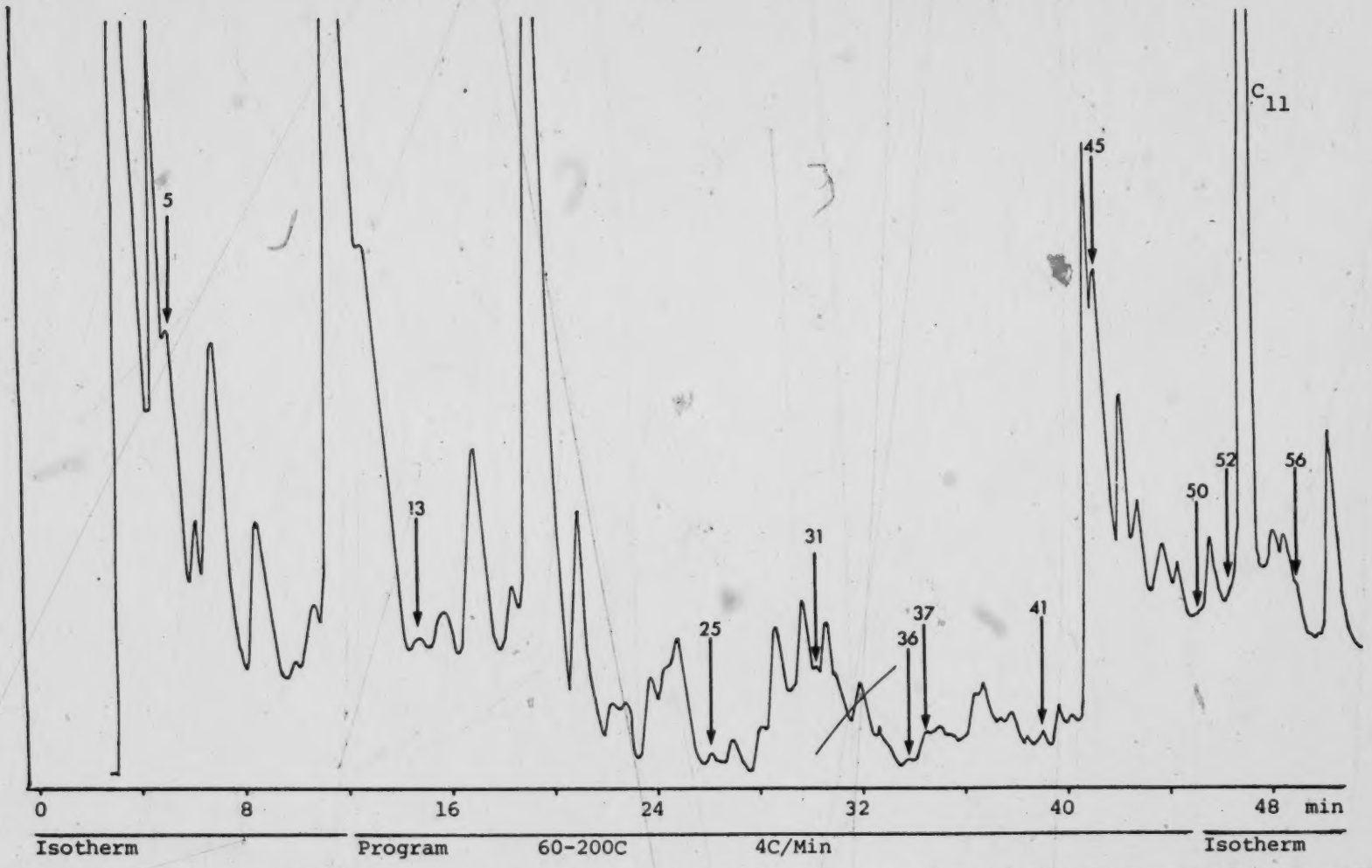


Fig. 15. Pyrogram of Nannizzia gypsea 1485 (+). Internal marker as shown is carbon-11 (methyl-caproate).

FREQUENCIES OF PROXIMITIES AT 0.01 LEVELS

LEVEL	NUMBER	TOTAL	REMAINDER	HISTOGRAM
0.01	0	0	2080	
0.02	0	0	2080	
0.03	0	0	2080	
0.04	0	0	2080	
0.05	0	0	2080	
0.06	5	5	2075	*
0.07	4	9	2071	*
0.08	8	17	2063	*
0.09	6	23	2057	*
0.10	6	29	2051	*
0.11	7	36	2044	*
0.12	15	51	2029	**
0.13	20	71	2009	***
0.14	15	86	1994	**
0.15	31	117	1963	*****
0.16	45	162	1918	*******
0.17	43	205	1875	*******
0.18	97	302	1778	**********
0.19	104	406	1674	**********
0.20	114	520	1560	**********
0.21	134	654	1426	**********
0.22	183	837	1243	**********
0.23	175	1012	1068	**********
0.24	177	1189	891	**********
0.25	182	1371	709	**********
0.26	171	1542	538	**********
0.27	145	1687	393	**********
0.28	105	1792	288	**********
0.29	97	1889	191	**********
0.30	56	1945	135	*********
0.31	51	1996	84	********
0.32	26	2022	58	****
0.33	21	2043	37	***
0.34	18	2061	19	***
0.35	11	2072	8	*
0.36	4	2076	4	*
0.37	2	2078	2	*
0.38	0	2078	2	
0.39	2	2080	0	*
0.40	0	2080	0	
0.41	0	2080	0	
0.42	0	2080	0	
0.43	0	2080	0	
0.44	0	2080	0	
0.45	0	2080	0	
0.46	0	2080	0	
0.47	0	2080	0	
0.48	0	2080	0	
0.49	0	2080	0	
0.50	0	2080	0	

Fig. 16. Computer print-out histogram of proximities from the equally weighted cluster analysis.

FIGURE 17

MDSCAL-Taxometric map of the relations among 21 strains of gymnoascaceous fungi. The clusters result from the equally weighted computer analysis of pyrogram data. The diameter of the circles represents the maximum distance between any pair of OTU's in the cluster. The lines connecting the margins of the circles represent the distance between the nearest neighbors in the two clusters. To fit the clusters into two dimensions, other distances may be distorted, as indicated by bent lines of the appropriate length and by straight lines of the appropriate length with dashed continuations. The arrows indicate the nearest neighbor to each cluster.

Cluster membership index: see Table VIII.

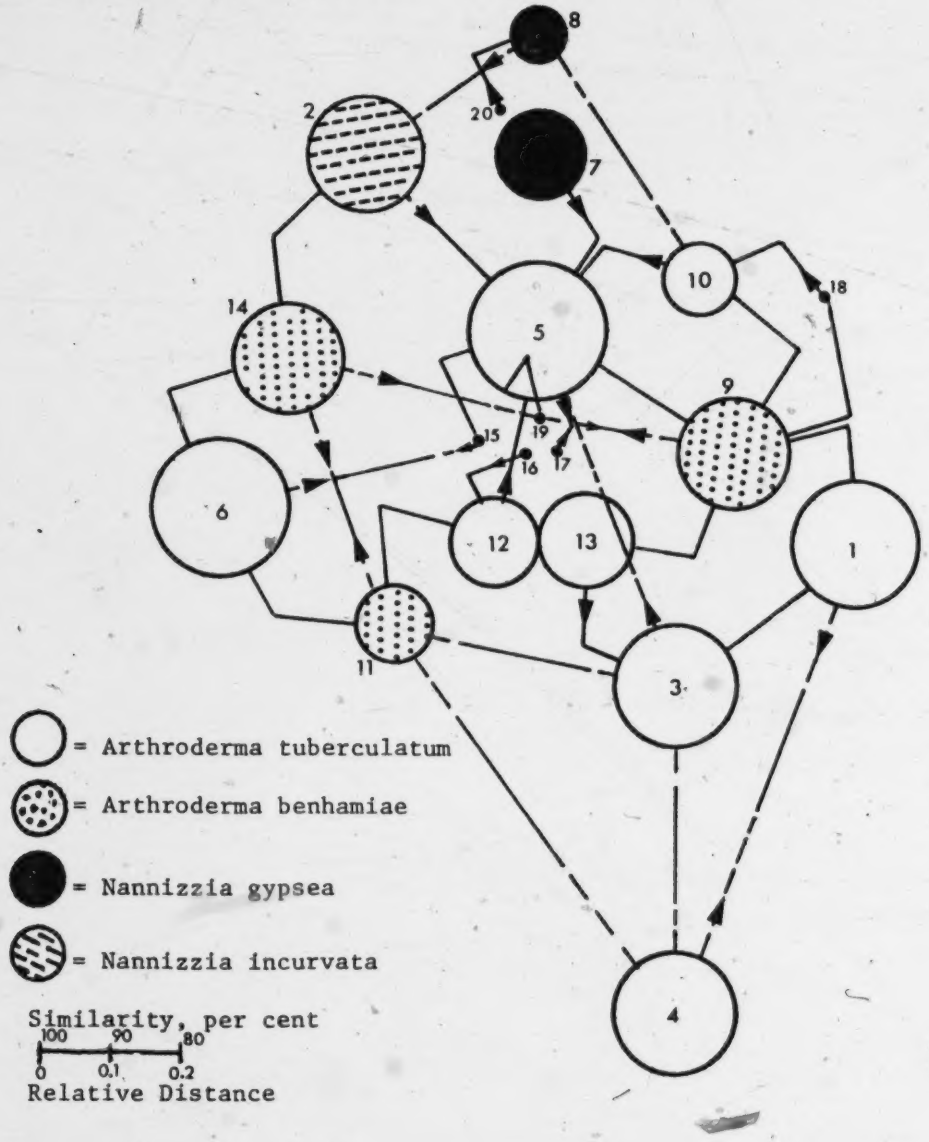


FIGURE 18

TAXMAP mapping procedure clusters of the equally weighted relations among 21 strains of gymnoascaceous fungi.

Cluster membership index: see Table VIII.

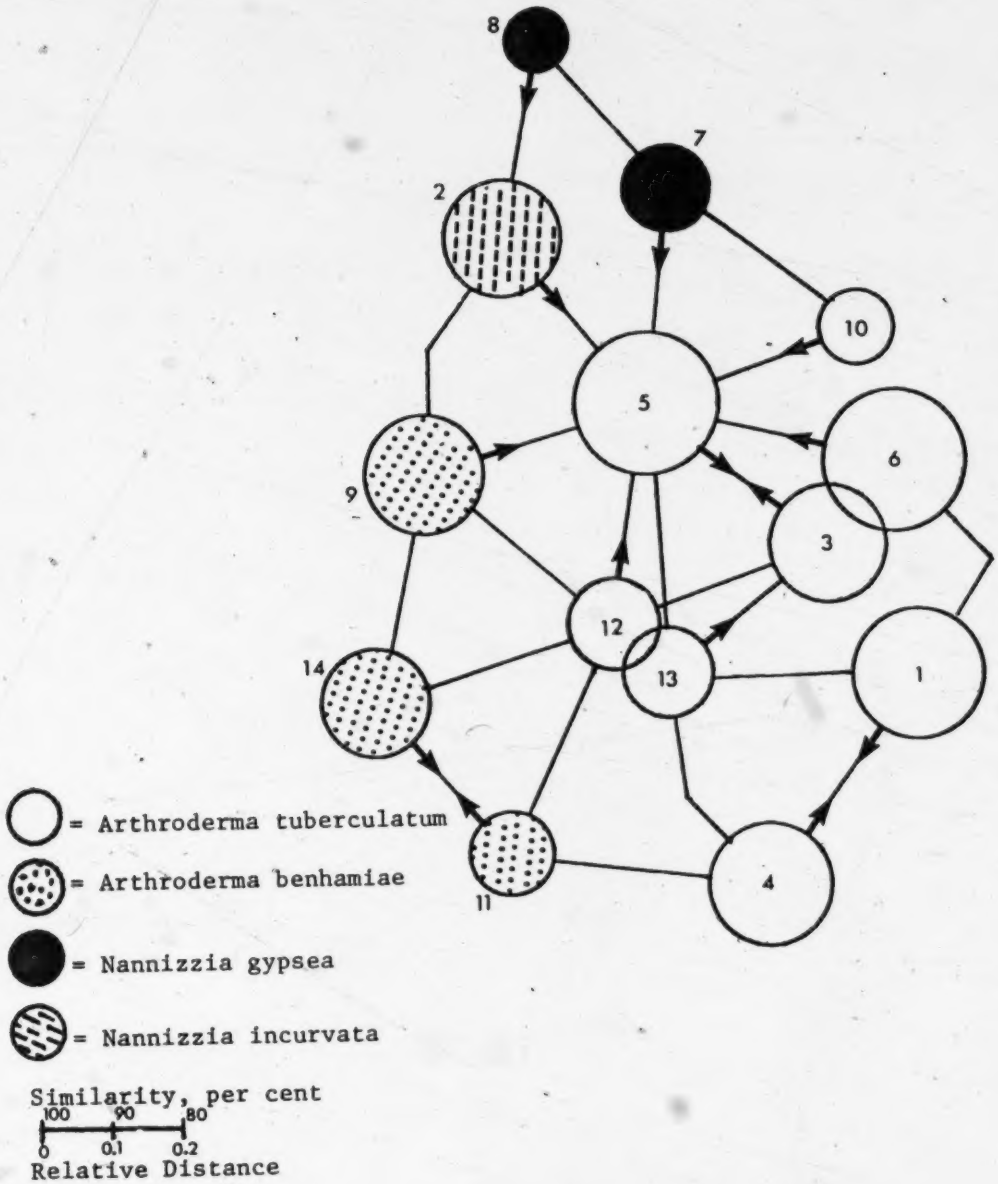


FIGURE 19

MDSCAL-Taxometric map of clusters of a weighted analysis among 21 strains of gymnoascaceous fungi, weighted according to the information content of each peak assuming equal intra-strain variation for all peaks.

Cluster membership index: see Table IX.

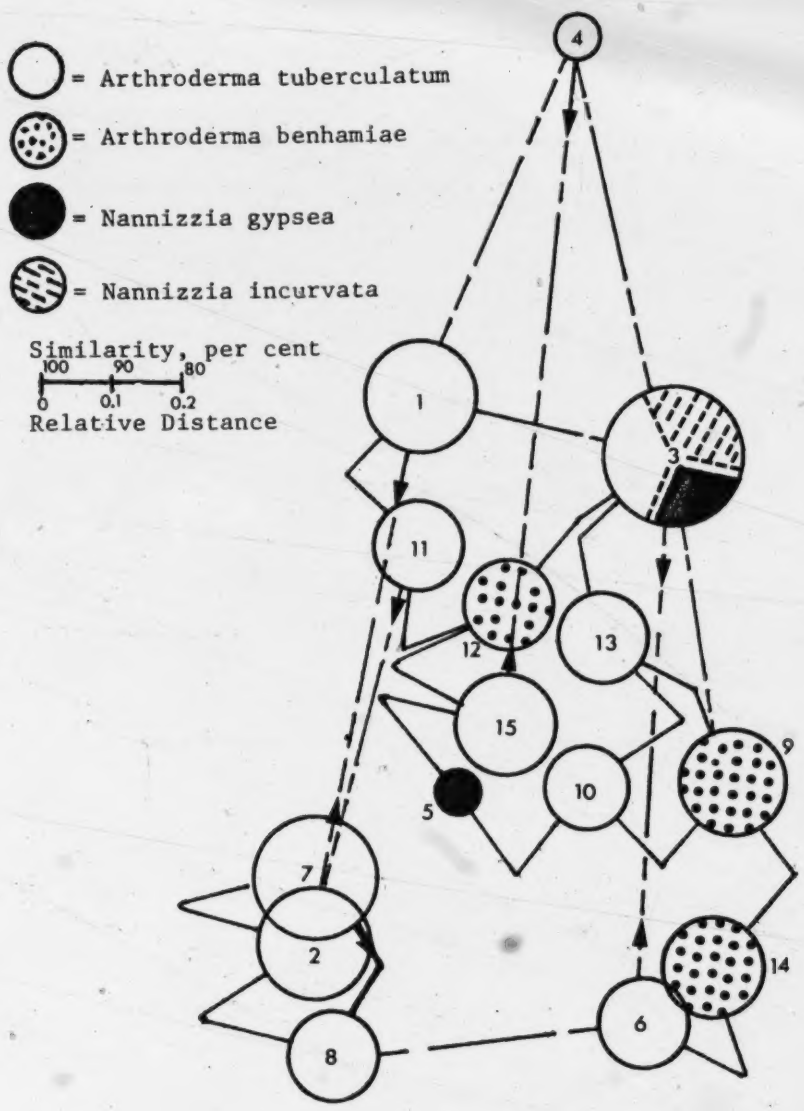


FIGURE 20

TAXMAP mapping procedure clusters of the same relations found for Fig. 19.

Cluster membership index: see Table IX.

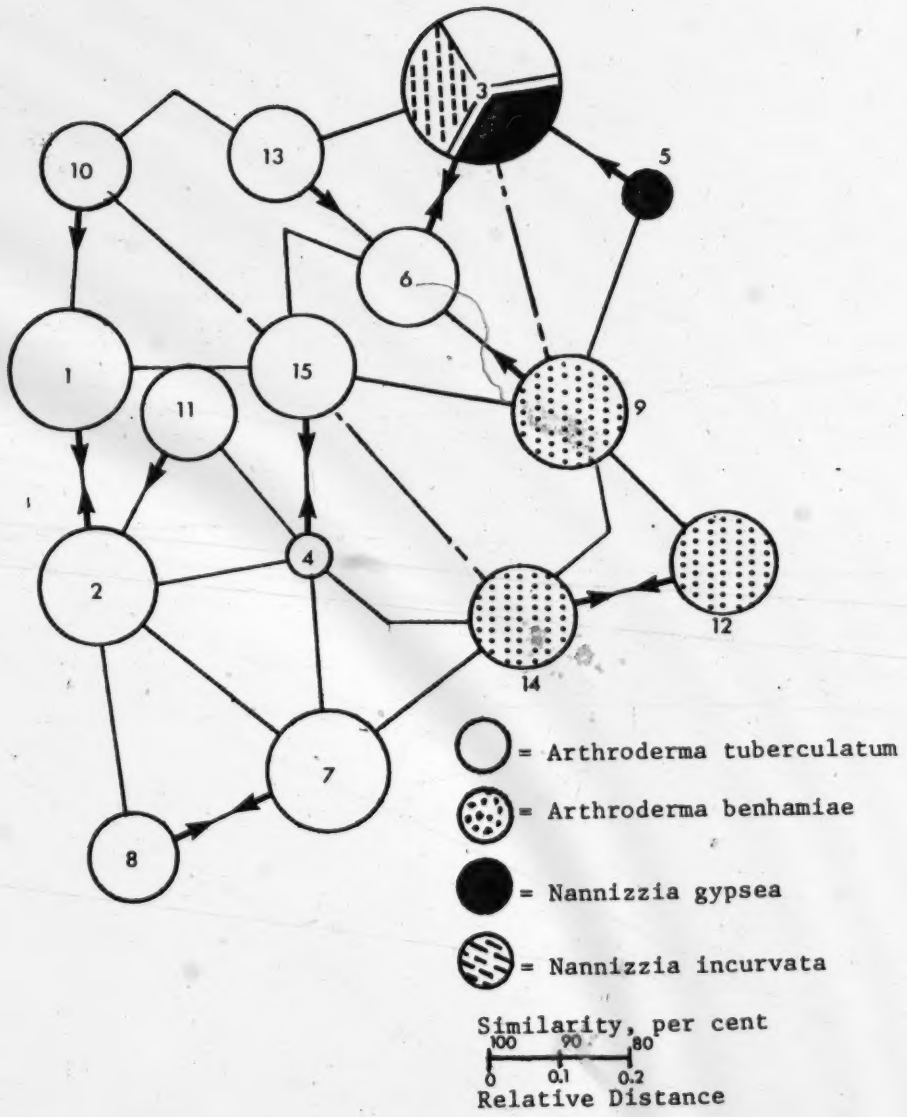


FIGURE 21

MDSCAL-Taxometric map of clusters of a weighted analysis among 21 strains of gymnoascaceous fungi, weighted for information content using the average intra-strain range for each peak. Included are 95% confidence intervals reflecting the variation among strain replicates.

Cluster membership index: see Table X.

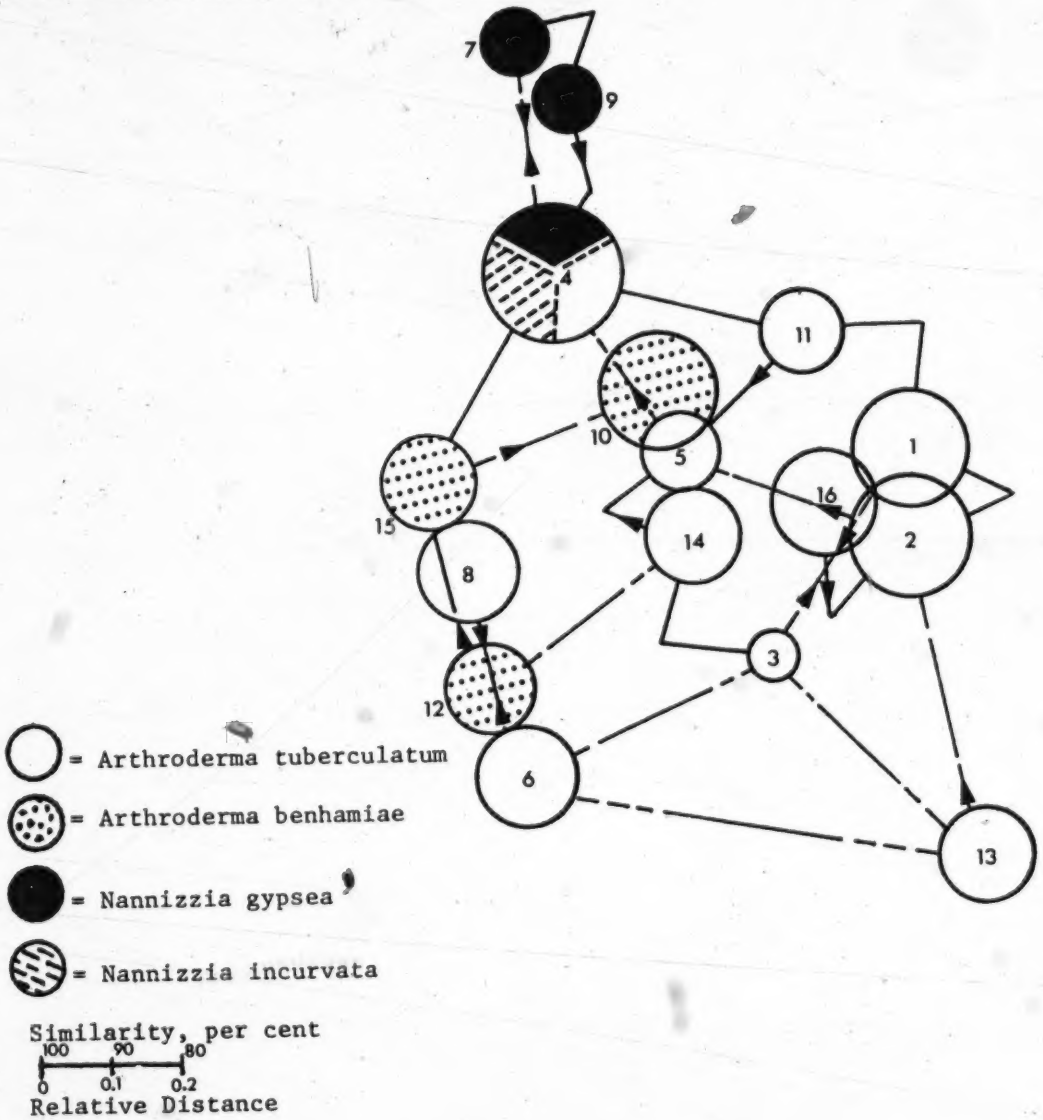
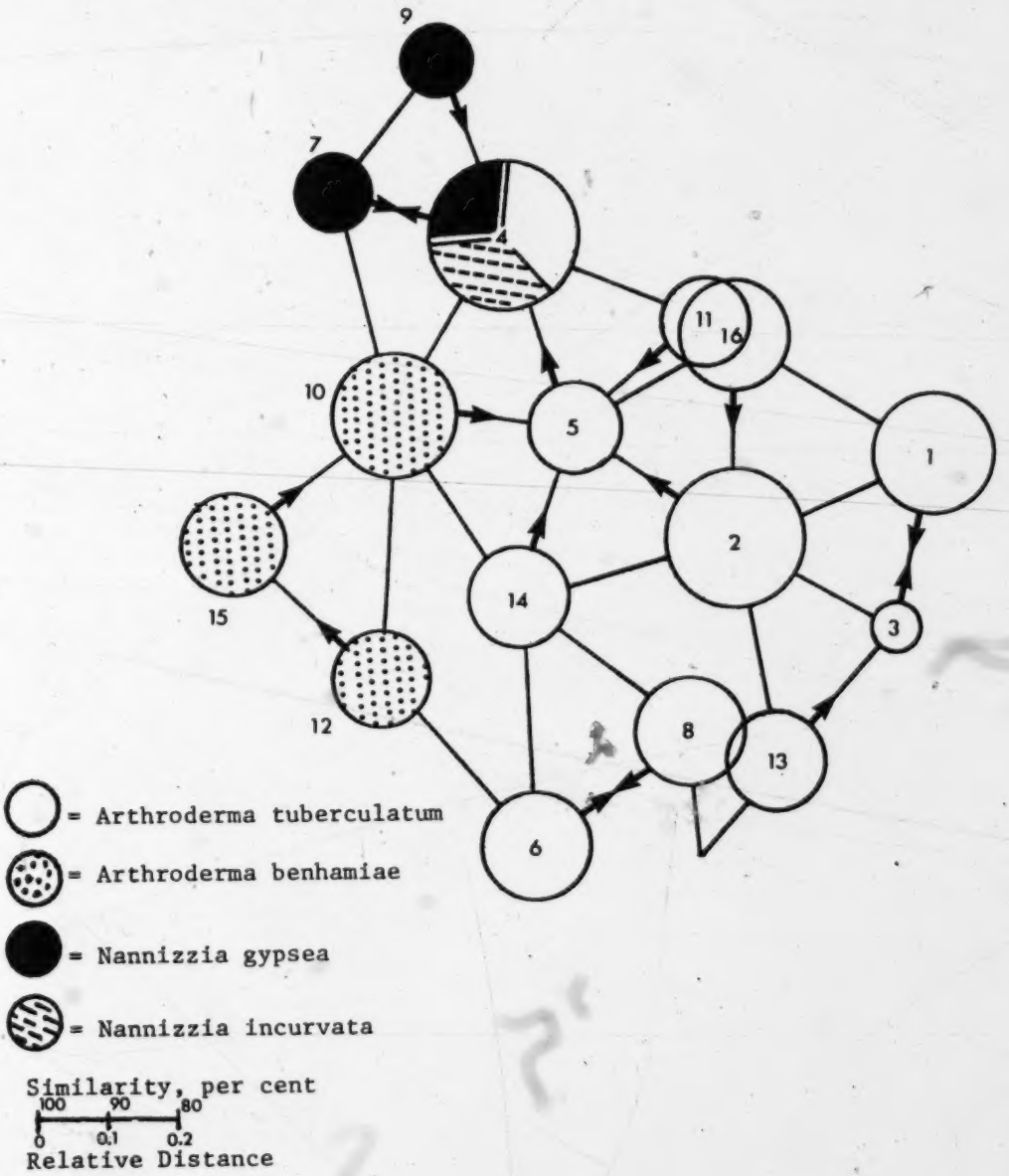


FIGURE 22

TAXMAP mapping procedure clusters of the relations found for Fig. 21. Included are 95% confidence intervals reflecting the variation among strain replicates.

Cluster membership index: see Table X.



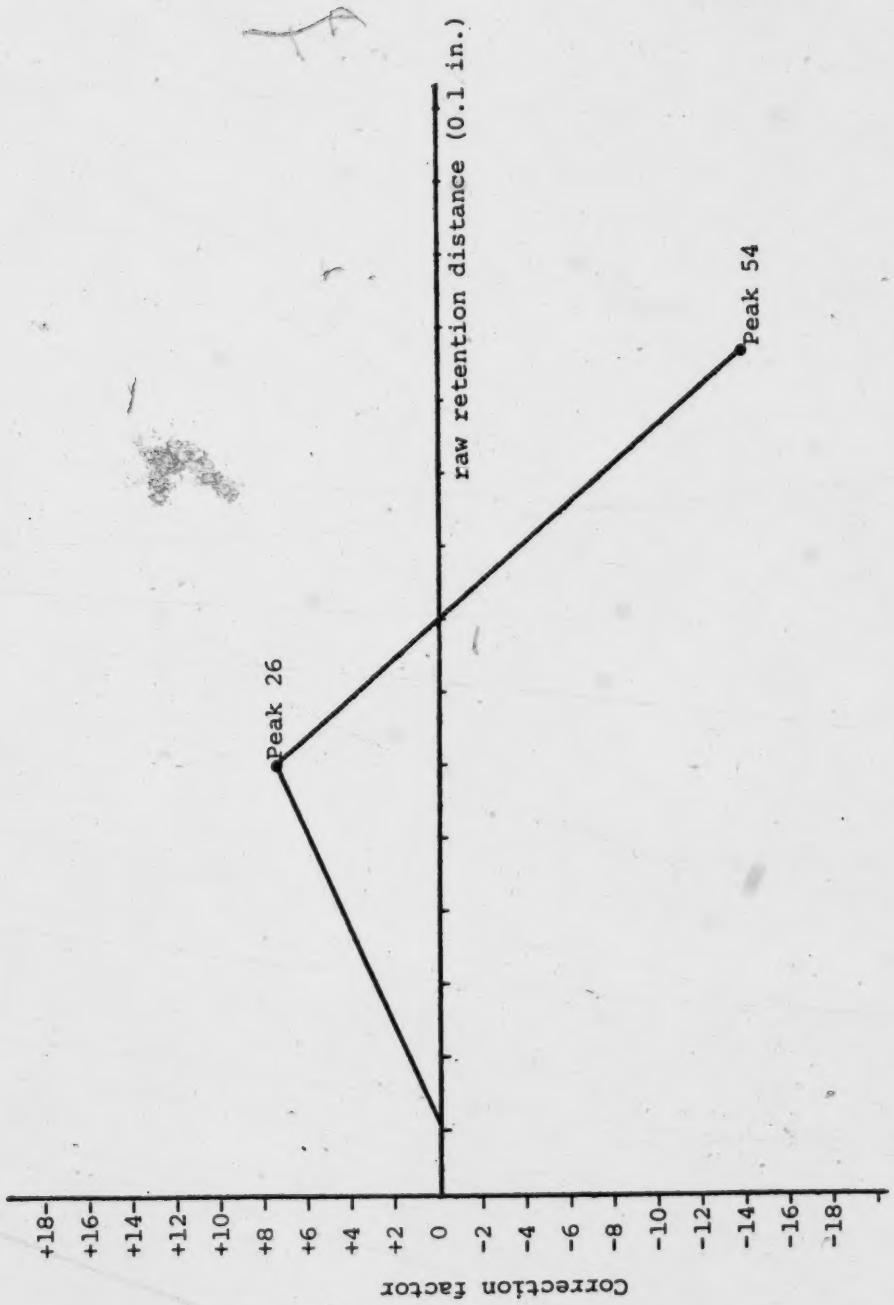


Fig. 23. Graph for determining correction factors to apply to peaks at retention times intermediate between the markers.

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